Nutrition and Cardiovascular Health in Renal Transplant Recipients

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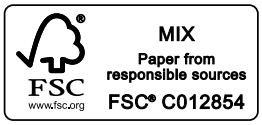

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Introduction and Aims of the Thesis

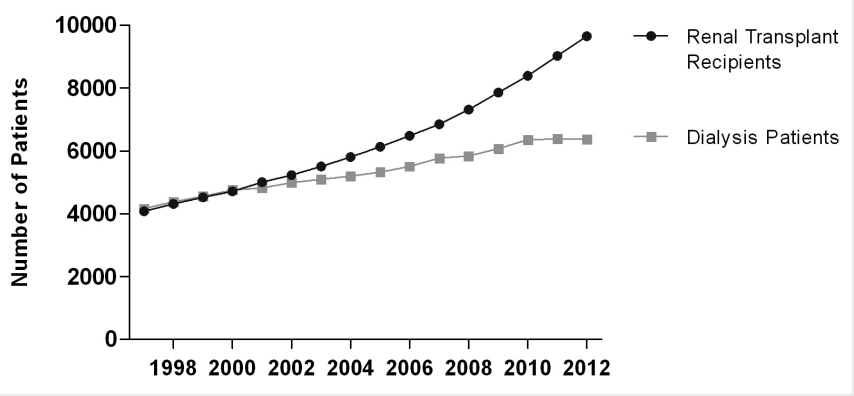
Introduction

The last few decades, the incidence and prevalence of end stage renal disease (ESRD) have been increasing steadily worldwide[1, 2]. In the Netherlands, the number of ESRD patients increased with about 10,000 patients over the last five years [3]. These growing numbers likely reflect the ageing population and the increasing rates of lifestyle diseases like hypertension, diabetes mellitus type 2 and obesity, all known to be risk factors for the development and progression of chronic kidney disease (CKD) [4-7]. Throughout the course of the renal disease, nutritional factors are relevant players in progression of kidney disease and its complications. This is partly due to the impairment of the renal capacity for excretion and metabolism of exogenous compounds that is consequent to renal function loss. Accordingly, increased susceptibility to the pathophysiological effects of exogenous compounds, including nutritional factors, is inherent to renal disease. The relationships between nutritional factors and renal disease and its complications, however, are complex, and only partly understood. Of note, these relationships are dynamic, evolving with the progression of kidney disease towards ESRD, and the different modes of renal replacement therapy. i.e dialysis and transplantation.

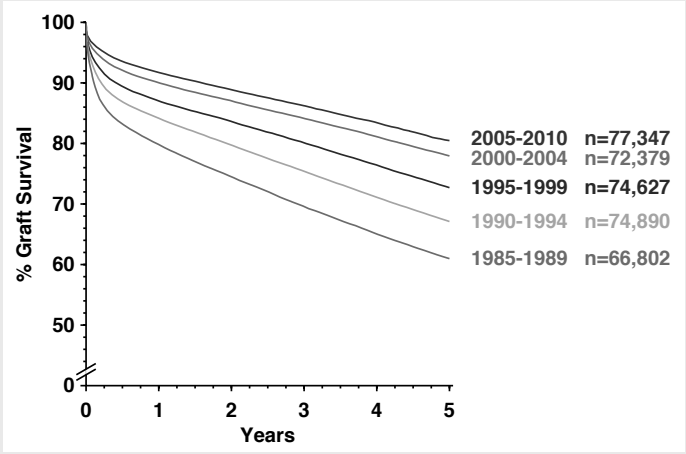
The majority of ESRD patients commences dialysis treatment as renal replacement therapy. However, despite the life-extending qualities of dialysis, it still may be inadequate on the long run for addressing the critical problems of persistent fluid overload, hypertension, heart failure, infections and related complications in this patient group. Mortality risk in dialysis patients is still unacceptably high with an annual death rate of 16 per 100 patient-years at risk [8], which means that only half of all dialysis patients is still alive three years after the start of dialysis. A main factor contributing to the high morbidity and mortality rates is the high prevalence of malnutrition and protein energy wasting in these patients [9, 10], which is closely related to uremia as a state of chronic inflammation [11]. However, inadequate intake is also involved. The requirements of protein in dialysis patients are high, while intake of protein and energy is usually low due to uremic toxicity, nausea, inflammation and psychosocial factors leading to loss of appetite [10]. Additionally, dialysis patients are subject to stringent and demanding dietary prescriptions, such as restricted intake of whole-grains, fruits and vegetables to limit phosphorus and potassium intake and prevent hyperphosphatemia and hyperkalemia. These restrictions, albeit of vital importance for risk reduction of morbidity and mortality, can pave the way to nutrient deficiencies and add to the status of malnutrition, eventually leading to a deterioration of the nutritional and clinical condition of dialysis patients.

For ESRD patients, renal transplantation is the preferred treatment as it ends the need for debilitating dialysis and improves both quality of life and life expectancy compared to patients on dialysis [8, 12, 13]. In the Netherlands, the number of ESRD patients with a functioning renal graft has increased considerably over the last decades and currently constitutes a group exceeding 10,000 patients [3] (*Figure 1*).

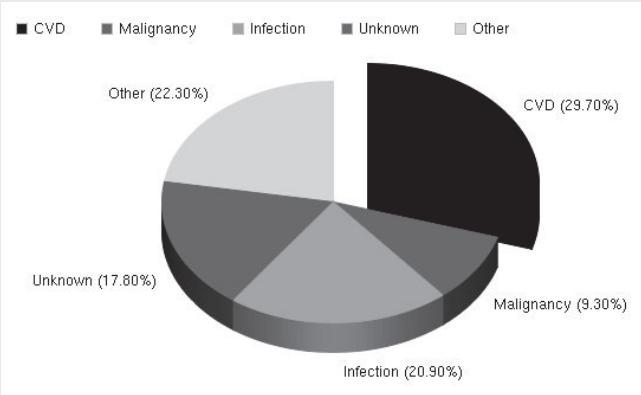
Figure 1 Number of Patients on Renal Replacement Therapy in the Netherlands



While deceased donors have been the main source for transplantation for many years, living kidney donors have become increasingly important, also allowing pre-emptive transplantation. The annual number of kidneys obtained from living donors has surpassed the annual number of deceased renal grafts in the Netherlands [14]. However, this only in part explains the increasing prevalence of ESRD patients with a functioning graft. A more valid explanation for the increasing numbers is the impressive improvement of one-year graft and patient survival, mainly as a result of improvement of surgical procedures, prevention of acute rejection, treatment of opportunistic infections and introduction of stronger and more effective immunosuppressive agents [15-17]. Over the last decades, an improved graft survival has been observed with a functioning graft for recipients of deceased and living kidney transplants at 90% and 95% respectively, compared to 40% in the 1970's [18]. Nevertheless, graft and also patient survival on the long run have not been concordant as can be seen in figure 2 where lines for graft survival over the different decennia run almost parallel.



Even after successful transplantation, morbidity and mortality rates are notably higher compared to the general population [19]. Of all patients who survive the first year after transplantation, 50% of renal grafts originating from deceased or living donors are lost within 12 and 25 years after transplantation respectively [2]. One of the main causes of graft loss is patient mortality with a functioning graft [20, 21]. Cardiovascular disease, including hypertension, endothelial dysfunction and atherosclerosis, continues to be the leading cause of death after renal transplantation (*Figure 3*) and prevalence has been estimated to be five times higher than in the general population [22-24]. Additionally, metabolic abnormalities and adverse effects of immunosuppressive medications significantly impact on the long-term morbidity and quality of life of renal transplant recipients. Previous studies observed a significant contribution of the chronic use of corticosteroids to post-transplant cardiovascular risk [25]. Similar adverse effects are attributed to immunosuppressive drugs such as calcineurin inhibitors and m-TOR inhibitors [26, 27]. As avoidance of immunosuppressive agents obviously is not feasible in patients with a renal graft, attention should be directed toward the search for other tools to help prevent post-transplant cardiovascular and metabolic diseases and to improve long-term graft and patient survival.



Nutritional factors could provide new targets for intervention in renal transplant recipients. At present, the majority of renal transplant recipients visiting the University Medical Center Groningen underwent dialysis prior to transplantation. They previously were committed to aforementioned strict dietary limitations and have been exposed to the high risk of malnutrition and associated conditions for varying periods of time, usually amounting to several years. After transplantation, abrupt changes in nutritional needs are experienced as most of these restrictions do no longer apply after transplant surgery, in proportion to the restoration of renal function. Due to the newly obtained freedom to indulge in many different foods, combined with reversal of the uremic state and an increased feeling of hunger caused by immunosuppressive drugs such as corticosteroids, excessive weight gain is commonly seen after renal transplantation [28-30]. Since overweight and obesity are well known risk factors for cardiovascular disease, in the general population [31] and particularly in patients already at risk like renal transplant recipients [32, 33], restriction of caloric intake should receive proper attention in clinical care. However, it undoubtedly is not merely food quantity that poses a threat to these patients. Cardiovascular as well as metabolic complications following transplantation, including hypertension, endothelial dysfunction, vascular calcification and low grade systemic acidosis, all conditions highly prevalent after renal transplantation, are likely to be influenced, at least in part, by the composition of the diet.

Over the last decades, various dietary factors and their effects on cardiovascular health have been studied in the general population. The 'Interheart' study in fifty-two countries revealed a positive correlation between cardiovascular risk and the Western diet, also after adjustment for body mass index [34] indicating that it indeed is not solely excessive body weight that is responsible for increased cardiovascular risk. One of the

most extensively studied nutritional risk factor for cardiovascular diseases is salt intake, not only in the general population [35, 36], but also in populations already at risk, such as patients with renal disease [37] and patients with established hypertension [38]. High salt intake is increasingly recognized as an important determinant of cardiovascular and renal damage. However, its role in renal transplant recipients is not yet clear. Another dietary factor that has been focus of numerous studies is dietary protein and its potential effect on blood pressure [39-41]. Evidence from a meta-analysis suggests a small beneficial influence of protein on blood pressure, especially for plant protein, in the general population [42]. In renal patients however, the beneficial role of protein intake is disputed as dietary protein, or its equivalent of amino acid infusion, can affect renal hemodynamics and induces high intra-glomerular pressure, subsequently leading to kidney damage and hypertension [43, 44]. For renal transplant recipients, it remains unknown what the optimal daily amount and optimal type of protein is.

Considering the above, despite the numerous studies that have been performed on the contribution of nutritional factors to physical conditions in a wide range of subgroups of the general population, renal transplant recipients have only sparsely been subject to nutritional studies. From the considerations mentioned above, it may be clear that renal transplant recipients constitute a very specific patient group in whom the impact of nutritional factors cannot simply be considered similar to the general population or 'common' chronic kidney disease patients. Therefore, point-to-point extrapolation of findings from studies on the role of nutrition in cardiovascular health in other can not groundlessly be done. As a result of the absence of any evidence regarding the long-term dietary requirements of stable renal transplant recipients, solid guidelines on nutrition are lacking. Therefore, it remains unclear, for medical practitioners as well as for renal transplant recipients, what the optimal composition of the diet should be to improve long-term outcomes in renal transplant recipients.

Aims of the Thesis

The aim of this thesis is to document the nutritional intake in a large cohort consisting of stable renal transplant recipients who have a functioning graft for at least one year and to compare their dietary habits with those in a healthy reference group. Second, we aim to investigate the associations of several nutritional factors with cardiovascular, metabolic and renal risk profiles long-term after renal transplantation, allowing identification of appropriate targets for interventions to help prevent morbidity and mortality in renal transplant recipients.

In chapter two we focus on dietary sodium intake which is an established risk factor for hypertension in the general population. We compare sodium intake, inferred from 24-hour urine samples, between renal transplant recipient and healthy controls and investigate the association of dietary sodium intake with blood pressure in our renal transplant cohort, under conditions of routine clinical care.
In chapter three we zoom in on protein intake, differentiating between total, animal and plant protein. We make use of a validated food frequency questionnaire and additionally measure urinary urea excretion, to assess dietary protein intake in our renal transplant recipients. Next, we analyze the association of protein intake with blood pressure and renal function, thereby differentiating between total, animal and plant protein.
In chapter four we elaborate on the acidifying properties of dietary protein and its potential association with acid-base homeostasis and cardiovascular risk parameters in renal transplant recipients. We estimate dietary acid load, applying diet based algorithms, and assess metabolic acid load by analysis of urinary net acid excretion. Subsequently, we aim to identify dietary factors contributing to metabolic acid load that could provide tools for improving acid-base balance in renal transplant recipients.
In chapter five we aim to shed some light on the association between sulfur metabolites and cardiovascular and metabolic parameters in renal transplant recipients. Conflicting hypotheses exist on the role of sulfur as it either might be harmful for acid-base homeostasis or, in contrast, be protective from a cardiovascular point of view through incorporation in the hydrogen sulfide metabolism. We assess intake of sulfur containing protein with a food frequency questionnaire and investigate its association with the two main urinary sulfur metabolites, sulfate and thiosulfate. Subsequently, we analyze the association of both sulfur metabolites with metabolic and cardiovascular parameters in our renal transplant recipients. In chapter six we investigate the association of endogenous synthesis of nitric oxide, a gaseous compound with a variety of protective properties, with various post-transplant cardiovascular risk parameters. We compare urinary NOx-excretion, reflecting systemic levels of nitric oxide, between renal transplant recipients and healthy controls. Next, we study whether potential differences in urinary NOx-excretion can be explained by differences in dietary habits. Third, we investigate whether levels of urinary NOx-excretion are associated with the cardiovascular risk profile in renal transplant recipients.
In chapter seven we determine intake of vitamin K and its association with vascular vitamin K status, defined as elevated plasma uncarboxylated matrix glutamate protein in sixty renal transplant recipients. Furthermore, we aim to identify dietary factors that are associated with vitamin K status which might provide new targets for intervention to improve vitamin K status after transplantation. Dietary intake is assessed with three-day dietary diaries instead of food frequency questionnaires to enhance accuracy.

In chapter eight the results of all studies are summarized. Furthermore, the implications of the findings in this thesis and the future perspectives are discussed.

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2

Sodium Intake and Blood Pressure in Renal Transplant Recipients

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Abstract

Background Hypertension is ubiquitous among renal transplant recipients (RTR) and a risk factor for graft failure and mortality. Sodium intake is a well established determinant of blood pressure (BP) in the general population. However, data in RTR are limited. International guidelines recommend a maximum daily sodium intake of 70mmol. We investigated sodium intake in RTR as compared to healthy controls, and its association with BP.

Methods We included 660 RTR (age 53±13 yrs, 58% male) and 201 healthy controls (age 54±11 yrs, 46% male). Sodium intake was assessed from 24h urine. The morning after completion of urine collection, BP was measured according to a strict protocol.

Results Urinary sodium excretion was 156±62 mmol/24h in RTR and 195±75 in controls (difference: P<0.001) and 95% of RTR had a UNaV above 70 mmol/24h. Systolic and diastolic BP were 136±18 and 82±11 mmHg respectively. Sodium intake was positively associated with SBP (β=0.042 mmHg per mmol/24h, P=0.002) and DBP (β=0.023 mmHg per mmol/24h, P=0.007), independent of potential confounders.

Conclusion RTR had a lower sodium intake than healthy controls, yet intake still exceeded current guidelines. Reduction of sodium intake to recommended amounts could reduce SBP by 4-5 mmHg. Better control of sodium intake may help to prevent graft failure and mortality due to hypertension among RTR.

Introduction

Renal transplantation is the preferred treatment for patients with end stage renal disease. Short term graft survival after clinical transplantation has improved progressively over the last decades. However, allograft and patient survival on the long run have not paralleled this improvement [1]. Leading cause of long term morbidity and mortality among renal transplant recipients (RTR) is cardiovascular disease (CVD) and therefore attention should be paid to its prevention and treatment [2, 3]. A major contributor to CVD is hypertension, which is very common after renal transplantation. Epidemiologic studies indicate that 50% to 90% of RTR either have hypertension (defined as BP higher than 140/90 mmHg) or are on antihypertensive medications [4, 5]. Prevention and treatment of hypertension should therefore receive proper attention.

Numerous studies have been performed on identification of factors that are positively associated with hypertension. High salt intake is an established risk factor for hypertension in the general population [6-9], but also in populations with chronic kidney disease (CKD) [10, 11]. Data on the association between sodium intake and BP in RTR, however, are sparse and inconclusive. The latter may be due to relatively small sample size of the studies [12-14] or a large time lag between collection of data on sodium intake and measurement of BP [15].

The Kidney Disease Outcomes Quality Initiative (K/DOQI) clinical practice guidelines on hypertension and antihypertensive agents in chronic kidney disease anticipate on a rising prevalence of hypertension among adults in the United States [16]. Like the Dietary Approach to Stop Hypertension (DASH) guideline, they advocate moderate consumption of sodium with a maximum daily intake of about 100 mmol per day, equaling 5.8 grams of sodium chloride per day [17]. Guidelines provided by the US Department of Health and Human Services (HHS) and the US department of Agriculture (USDA) promote an even more strict regimen of no more than 1,500 mg/d (~70 mmol/d) in individuals with hypertension, middle-aged and older adults [18, 19]. One older study reported that sodium intake in RTR is higher rather than lower than in healthy controls and usually exceeds even the conservative limit of 100 mmol per day [14].

The aim of our study, therefore, was to compare sodium intake in RTR with that in healthy controls and to investigate the association of dietary sodium intake with blood pressure in a large single center RTR cohort study under conditions of routine clinical care.

Methods

Subjects

We invited all adult RTR with a functioning graft for at least one year who visited our outpatient clinic between 2008 and 2010. A group consisting of 660 out of 702 initially invited RTR signed written informed consent. As a healthy reference group, we included all 201 subjects evaluated for living kidney donation in our center during the same period. None had a history of kidney disease, diabetes or cardiovascular events. Hypertension, if present, was regulated with a maximum of one antihypertensive drug. All participants signed informed consent. The Institutional Review Board approved the study protocol (METc 2008/186) which was in adherence to the Declaration of Helsinki.

Data collection

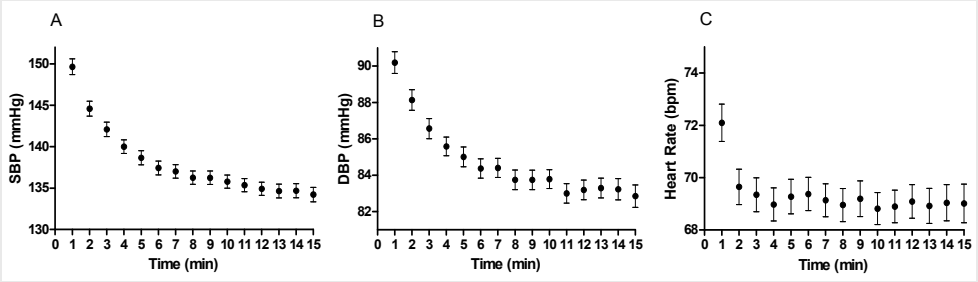
Height, weight and waist circumference were measured at the day of the visit to the outpatient clinic. Body Mass Index (BMI) was calculated as weight divided by height squared (kg/m^2) and Body Surface Area (BSA) was estimated applying the universally adopted formula of DuBois and DuBois [20]. Information on medication use was derived from patient records and data on smoking behavior was inquired with a questionnaire. RTR were classified as current smokers, former smokers, or never smokers.

Blood was drawn after an 8-12h overnight fasting period in the morning after completion of the 24h urine collection. To assure adequate urine collection, both RTR and healthy controls were carefully instructed. Subjects were informed to discard their morning urine specimen, collect all subsequent urine through the next 24 hours and include the next morning's first specimen of the day of their visit to the outpatient clinic. Renal function was assessed by 24h urinary creatinine clearance (ml/min) calculated as time-factored urinary creatinine concentration (mg/min) divided by plasma creatinine concentration (mg/mL). Daily salt intake was assessed on basis of 24h urinary sodium excretion (UNaV), since urinary sodium excretion largely equals sodium intake, when people are in steady-state [21, 22]. Adequacy of 24h collection was assessed by comparing the actual 24h creatinine-excretion rate (CER) with creatinine excretion as predicted by body dimensions, age and gender [23]. In secondary analyses, urine samples deviating more than 30% from the predicted CER were excluded to investigate the association between UNaV and BP in RTR without possible noise due to inadequate urine collections.

Fasting BP (mmHg) was measured according to a standard protocol, which has been used in clinical studies from our department [24, 25] and in the large epidemiological PREVEND study (n = 8,592) [26-28]. BP was measured with one and the same semi-automatic device (Dinamap® 1846, Critikon, Tampa, USA) and all measurements were performed by the same investigator. To prevent white-coat effects, [29], participants were

left alone in a room in half-sitting position while BP and heart rate were measured every minute for fifteen minutes. The average of the last three values was taken as BP value in our study. The validity of this procedure is shown in Figure 1, depicting mean BP and heart rate over time. SBP and DBP of the averaged last three measurements were respectively 14 mmHg and 7 mmHg lower than the first measurement (both $p<0.0001$). BP reaches a plateau after about ten measurements, equivalent to ten minutes. The day-to-day coefficient of variation for BP evaluated in this way was 2.0% for SBP and 4.3% for DBP. Mean arterial pressure (MAP) was calculated as twice the DBP plus SBP divided by three, approximating the average arterial pressure during a single cardiac cycle [30]. Measurements were performed while patients were on their regular medication, including anti-hypertensive drugs at trough. All measurements were performed at the morning of the visit to the out-patient clinic.

Figure 1. Course of systolic blood pressure, diastolic blood pressure and heart rate during 15 minutes of automated blood pressure measurements. A: Systolic blood pressure; B: Diastolic blood pressure; C: Heart rate. Values are given as mean with standard error.



Statistical analysis

Analyses were performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL). Normality was tested with the Kolmogorov-Smirnov test. Skewed data were normalized by logarithmic transformation in analyses. Parametric variables are expressed as mean±SD, whereas non-parametric variables are given as median [interquartile range]. Differences between RTR and healthy controls were tested with the t-test for independent samples, the Mann-Whitney U test or the chi-square test. To visualize associations of UNaV with BP in RTR, this study population was divided into gender- stratified tertiles of MAP. P-values for differences between these tertiles were obtained using the ANOVA, Kruskal-Wallis test or chi-square test. Linear trends of variables over the tertiles of

MAP were analyzed applying univariate linear regression analysis, to identify potential confounders of the association between sodium intake and BP. The association of sodium intake with MAP, SBP and DBP was investigated with multivariate linear regression analyses, with adjustments for age and gender (model 1), subsequently for waist circumference, renal function and smoking behavior (model 2) and plasma potassium concentration, urinary potassium excretion, proteinuria, HbA1c (continuous) and use of antihypertensives (ordinal) and calcineurine inhibitors (dichotomous) (model 3). A two-sided P value less than 0.05 was considered to indicate statistical significance.

Results

The characteristics of RTR and healthy controls are shown in *table 1*. The two groups were similar with respect to age, BMI and BSA. Men were overrepresented in the RTR-group compared to the healthy controls.

Sodium intake, as inferred from 24h urine sodium excretion, was significantly lower in the RTR group than in the healthy controls (156 ± 62 mmol/24h vs. 195 ± 75 mmol/24h, $p<0.0001$). Sodium intake in the RTR-group ranged from 19 to more than 300 mmol per day. Of all RTR, 85% had a sodium intake above 100 mmol per day and 95% had a sodium intake exceeding 70 mmol per day as recommended in international guidelines. One fifth of the patients had a sodium intake over 200 mmol per day.

As anticipated, creatinine clearance was significantly lower in RTR than in healthy subjects (65±26 vs. 123±37 ml/min; $p<0.0001$). Blood pressure was significantly higher in RTR than in controls (MAP 107±15 vs 95±17, $p<0.001$; SBP 136±18 vs. 125±15 mmHg, $p<0.0001$; DBP 83±11 vs. 76±9; $p<0.0001$), despite significantly more use of anti-hypertensive drugs in RTR, as shown in *table 2*. The majority of RTR (91%) either had hypertension or was on antihypertensive medications. Seventy two (11%) RTR were not using any anti-hypertensive drugs, while 198 (30%) RTR used one, 231 (35%) two and 159 (24%) three or more different anti-hypertensive drugs.

Table 1 Patient characteristics of study participants at the day of their visit to the out-patient clinic

Characteristics	Control Subjects (n = 201)	RTR (n = 660)	P
Demographics			
Age (years)	53.5 ± 10.6	53.3 ± 12.6	0.9
Male gender, n (%)	92 (46)	377 (57)	<0.01
Waist Circumference, men (cm)	94 ± 9	102 ± 13	<0.001
Waist Circumference, women (cm)	89 ± 10	95 ± 16	<0.001
BMI (kg/m^2)	26.0 ± 3.5	26.6 ± 4.8	0.1
BSA (m^2)	1.95 ± 0.20	1.94 ± 0.22	0.7
Smoking behavior			
Never, n (%)	105 (52)	277 (42)	0.03
Current, n (%)	48 (24)	79 (12)	<0.001
Ex-smoker, n (%)	48 (24)	304 (46)	<0.001
Medication use			
Patients on antihypertensives, n (%)	17 (8)	588 (89)	<0.001
Amount of antihypertensives used (n)	0 [0-0]	2 [1-2]	<0.001
Statins, n (%)	10 (5)	357 (54)	<0.001
Antidiabetic Drugs, n (%)	0 (0)	106 (16)	<0.001
Hemodynamic parameters			
Heart Rate (bpm)	67 ± 10	68 ± 11	0.004
Systolic Blood Pressure (mmHg)	125 ± 15	136 ± 18	<0.001
Diastolic Blood Pressure (mmHg)	76 ± 9	83 ± 11	<0.001
Mean Arterial Pressure (mmHg)	95 ± 17	107 ± 15	<0.001
Renal Function			
Serum Creatinine (umol/L)	72 [65-82]	125 [99-160]	<0.001
Creatinine Clearance (ml/min)	123 ± 37	65 ± 26	<0.001
Albuminuria (mg/24h)	5.4 [3.1-9.2]	39.0 [9.6-193.9]	<0.001
Urinary Protein Excretion (g/24h)	0 [0.0-0.2]	0.2 [0.0-0.4]	<0.001
Serum Parameters			
Glucose (mmol/L)	5.3 [5.0-5.6]	5.2 [4.8-6.0]	0.4
hsCRP (mg/L)	1.2 [0.6-2.4]	1.6 [0.7-4.6]	<0.001
Cholesterol (mmol/L)	5.34 ± 1.05	5.12 ± 1.12	0.01
HbA1c (%)	5.7 ± 2.2	6.0 ± 0.9	0.003
Urinary Parameters			
Sodium excretion (mmol/24h)	195 ± 75	156 ± 62	<0.001
Urea excretion (mmol/24h)	402 ± 119	387 ± 111	0.10
Chloride excretion (mmol/24h)	198 ± 72	146 ± 59	<0.001
Potassium excretion (mmol/24h)	92 ± 29	73 ± 24	<0.001
Calcium excretion (mmol/24h)	4.8 [3.4-6.7]	2.4 [1.1-3.9]	<0.001
Creatinine excretion (mmol/24h)	13.1 ± 4.2	11.6 ±3.4	<0.001

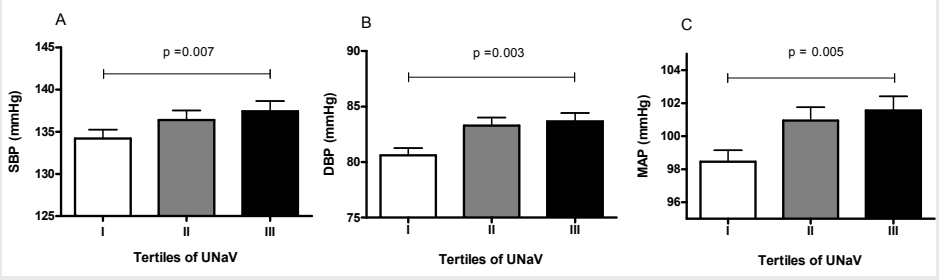
Table 2 Classes and number of medication used by the RTR

Type of medication	Number of RTR (%)
Antihypertensive drugs	
AII-antagonist	99 (15)
ACE-inhibitor	218 (33)
Beta blockers	416 (63)
Diuretics	271 (41)
Loop diuretics	136 (21)
Thiazide diuretics	106 (16)
Potassium-sparing diuretics	3 (<1)
Combination	27 (4)
Calcium entry blockers	152 (23)
Number of antihypertensive drugs	
None	72(11)
1 class	198 (30)
2 different drugs	231 (35)
3 or more different drugs	159 (24)
Anti-diabetic drugs	
Sulfonylurea	26 (4)
Biguanide	27 (4)
Insulin	65 (10)
Immunosuppressive drugs	
Proliferation inhibitor	548 (83)
Calcineurine inhibitor	376 (57)
Prednisolone dose (mg/day)	10 [7.5-10.0]

In RTR, MAP was significantly higher in men than in women (108±14 mmHg vs 105±16 mmHg, p=0.02). Therefore, further characteristics are presented according to gender stratified tertiles of MAP (table 3). With increasing BP, RTR were older and had higher BMI and BSA. Smoking behavior did not differ between tertiles. Regarding use of medication, more RTR were on antihypertensive medication in the highest tertile of MAP. Furthermore, use of anti diabetic drugs and calcineurin inhibitors gradually increased over the tertiles. Plasma creatinine and proteinuria differed significantly between tertiles (both p<0.001), however, creatinine clearance was similar. UNaV increased along with

increasing BP. In the highest tertile, mean UNaV excretion was 165 mmol, compared to 152 mmol in the lowest tertile (p<0.001). Figure 2 shows the mean BP values across gender stratified tertiles of UNaV. SBP, DBP and MAP were all significantly higher in the highest tertile compared to the lowest tertile of urinary sodium excretion (138 vs 134 mmHg, p=0.01; 84 vs 81 mmHg, p=0.003 and 102 vs 98 mmHg; p=0.005 respectively). Urinary volume increased with increasing tertiles of urinary sodium excretion (2209±734 mL, 2244±749 mL and 2660±847 mL respectively, p<0.001). The same was true for urinary sodium concentrations (48±19 mmol/L, 67±23 mmol/L and 91±33 mmol/L respectively, p<0.001).

Figure 2. Systolic blood pressure, diastolic blood pressure and mean arterial pressure of renal transplant recipients according to gender stratified tertiles of urinary sodium excretion. A: systolic blood pressure; B: diastolic blood pressure; C: mean arterial pressure. Values are mean with standard deviation. UnaV=urinary sodium excretion.



Associations between UNaV and BP in RTR, analyzed as continuous variables, are shown in table 4. With adjustment for age and gender, βs for the association with MAP, SBP and DBP were 0.030 (p<0.001), 0.038 (p=0.001) and 0.026 (p<0.001) respectively. Additional adjustment for confounders as accounted for in the third model, revealed betas of 0.030 (p=0.002), 0.042 (p=0.002) and 0.023 (p=0.007) for the association of UNaV with MAP, SBP and DBP respectively.

Table 3 Patient characteristics according to gender-stratified tertiles of Mean Arterial Pressure (MAP; mmHg)

	Gender-Stratified Tertiles of MAP (mmHg)			
	1	2	3	P
	n=218	n=223	n=219	
MAP (mmHg)	92 ± 8	106 ± 4	123 ± 10	
Demographics				
Gender (% male)	57%	57%	56%	NS
Age (years)	52 ± 14	53 ± 13	55 ± 12	0.02
Waist Circumference men (cm)	100 ± 14	100 ± 13	104 ± 13	0.002
Waist Circumference women (cm)	94 ± 15	94 ± 15	96 ± 17	0.5
BMI (kg/m^2)	26.1 ± 4.6	26.5 ± 5.1	27.0 ± 4.6	0.004
BSA (m^2)	1.92 ± 0.22	1.94 ± 0.21	1.95 ± 0.23	0.001
Smoking behavior				
Never (%)	43%	44%	39%	0.2
Current (%)	11%	12%	14%	0.3
Former (%)	46%	45%	47%	0.5
Time after transplantation (y)	5.9 [3.0-15.3]	5.7 [2.00-11.6]	5.2 [1.4-10.9]	0.02
Medication use				
Antihypertensive drugs, n (%)	190 (87)	194 (87)	197 (90)	0.04
ACEi or AII antagonist, n (%)	105 (48)	94 (42)	110 (50)	0.5
β-blocker, n (%)	135 (62)	140 (63)	142 (65)	0.16
Diuretics, n (%)	76 (35)	87 (39)	105 (48)	0.02
Number of antihypertensives	2 [1-2]	2 [1-2]	2 [1-3]	0.13
Statins, n (%)	111 (51)	114 (51)	131 (60)	0.16
Antidiabetic drugs, n (%)	20 (9)	40 (18)	44 (20)	0.01
Proliferation inhibitor, n (%)	203 (93)	185 (83)	182 (83)	0.9
CNI, n (%)	107 (49)	127 (57)	140 (64)	<0.001
Hemodynamic parameters				
Heart rate (bpm)	69 ± 14	69 ± 11	68 ± 11	0.16
Systolic Blood Pressure (mmHg)	121 ± 13	135 ± 10	152 ± 14	<0.001
Diastolic Blood Pressure (mmHg)	73 ± 8	83 ± 6	92 ± 9	<0.001
Renal Function				
Serum Creatinine (umol/L)	124 [98-155]	122 [97-153]	132 [102-175]	<0.001
Creatinine Clearance (ml/min)	65 ± 24	68 ± 26	63 ± 27	0.18
Protein excretion (g/24h)	0.2 [0.0-0.3]	0.2 [0.0-0.3]	0.3 [0.0-0.6]	0.001
Proteinuria ≥ 0.5 g/24h (%)	21%	18%	32%	<0.001

Serum Parameters				
Sodium (mmol/L)	140 ± 3	141 ± 3	141 ± 3	0.03
Potassium (mmol/L)	4.0 ± 0.5	3.9 ± 0.4	4.0 ± 0.5	0.32
Chloride (mmol/L)	105 ± 3	105 ± 3	106 ± 4	0.19
Urea (mmol/L)	9.4 [7.2-13.2]	9.0 [6.9-11.4]	10.4 [7.5-15.6]	0.001
Glucose (mmol/L)	5.2 [4.7-6.0]	5.3 [4.8-6.1]	5.3 [4.8-6.1]	0.4
hsCRP (mg/L)	1.8 [0.7-5.1]	1.6 [0.8-5.1]	1.6 [0.8-3.7]	0.15
Cholesterol (mmol/L)	5.0 ± 1.1	5.0 ± 1.1	5.3 ± 1.1	0.006
HbA1c (%)	5.7 [5.5-6.0]	5.8 [5.5-6.2]	5.9 [5.5-6.4]	0.02
Urinary Parameters				
Urine volume (mL)	2437 ± 816	2445 ± 768	2435 ± 812	0.99
Sodium excretion (mmol/24h)	152 ± 63	153 ± 61	165 ± 60	<0.001
Chloride excretion (mmol/24h)	143 ± 60	144 ± 59	154 ± 57	<0.001
Urea excretion (mmol/24h)	388 ± 115	378 ± 106	393 ± 113	0.15
Creatinine excretion (mmol/24h)	11.5 ± 3.5	11.5 ± 3.3	11.6 ± 3.5	0.17
Potassium excretion (mmol/24h)	73 ± 24	72 ± 25	73 ± 24	0.37
Calcium excretion (mmol/24h)	1.98 [1.2-3.7]	2.6 [1.3-3.9]	2.5 [1.0-4.2]	0.9

Adequacy of urine collection

In secondary analyses, reliability and completeness of the 24h urine collections of RTR was evaluated by comparing 24h creatinine excretion with estimated creatinine excretion applying the equation recently proposed by Ix et al. Samples deviating more than 30% were excluded and analyses for the association between UNaV and BP were repeated. Based on all separate expected urinary creatinine clearances, 77% of all urine samples were considered to be collected properly. Consequently, the sample size of our study was reduced noticeably, leaving us with 502 samples. Nevertheless, associations of UNaV with MAP, SBP and DBP became slightly stronger, with βs of 0.033 (P<0.001), 0.045 (p=0.001) and 0.026 (p=0.003) respectively (data not shown).

Table 4 Regression coefficients for the association of UNaV with MAP, SBP and DBP (change in mmHg per mmol sodium/24h)

Model	Mean Arterial Pressure			Systolic Blood Pressure			Diastolic Blood Pressure		
	β	95% CI for β	p	β	95% CI for β	p	β	95% CI for β	p
1	0.030	0.015-0.047	<0.001	0.038	0.018-0.033	0.001	0.026	0.012-0.041	<0.001
2	0.029	0.009-0.044	0.002	0.038	0.013-0.063	0.003	0.024	0.007-0.037	0.005
3	0.030	0.011-0.047	0.002	0.042	0.015-0.067	0.002	0.023	0.006-0.039	0.007

Model 1: adjusted for age and gender
Model 2: as 1, additionally adjusted for waist circumference, renal function and smoking behavior
Model 3: as 2, additionally adjusted for plasma potassium level, urinary potassium excretion, proteinuria, HbA1c and use of CNI and antihypertensives

Discussion

Hypertension is highly prevalent among RTR and contributes to cardiovascular disease and mortality in these patients [31, 32]. In line, in our population the large majority of RTR had hypertension, defined as a BP higher than 140/90 mmHg or the use of antihypertensive medications. At variance with prior reports [12-15] we found that sodium intake in RTR was lower than in control subjects with similar body composition. Nevertheless, in 95% of the patients sodium intake still exceeded the 70 mmol as recommended in current guidelines. In RTR a significant association between sodium intake and BP was observed, for both SBP and DBP, independent of potential confounders. As for RTR, it can be inferred that in our population, compliance with the maximally recommend daily intake of 70 mmol would have reduced mean SBP and DBP with approximately 5 mmHg and 3 mmHg respectively.

This study is the first to demonstrate a positive association between sodium intake and BP in RTR. These data suggest that better control of sodium intake can lead to clinically relevant improvement of BP in RTR.

The finding that RTR used less sodium than the controls (despite similar age and body composition), may be due to the fact that they were under medical supervision, including regular attention for general lifestyle advice. As for RTR, the large majority of patients has been under close dietary supervision during the period of end stage renal disease preceding renal transplantation. The lower potassium excretion suggests that the dietary habits of the RTR still partly reflect the dietary restrictions during dialysis. Nevertheless, mean daily sodium intake in RTR still exceeded current international guidelines, as was also found in several previous studies reporting mean 24h urinary sodium excretion rates of 178, 163, 224 and 165 mmol/24h respectively [33-36]. This clearly leaves room for improvement.

Considering the achievement of sodium restriction goals, RTR performed comparably as patients with CKD, in whom sodium intake is generally more or less equal to the general population, as reviewed elsewhere [37, 38].

We acknowledge that this study has limitations. First, it is a cross-sectional, epidemiologic study. Therefore, no causal relationship can be proven. Subjects with a high sodium intake for instance, may have other dietary or lifestyle habits than subjects with a lower sodium intake which might influence BP. The observation that the association remains significant in the multivariate regression analysis indicated that it is independent of the tested potential confounders, including age, gender and medication that could alter sodium handling and BP. It is, however, possible that residual confounding remained even in multivariate analyses, because it is hard, if not impossible, to adjust for (the severity of) each confounder. Of the RTR, 41% used oral diuretics, which can acutely

influence urinary sodium excretion. However, it is not likely that interference occurs of the association between urinary sodium excretion and BP in RTR. Because these diuretics are chronic medication in RTR who are at least one year after transplantation, sodium balance will be in steady-state while on medication, with sodium excretion representing sodium intake.
Furthermore, our study population consisted predominantly of Caucasian people, making extrapolation of our results to other ethnicities difficult. Nevertheless, the large size of the study population involved, and the performance of the measurements in a standardized manner renders our finding of a positive association between sodium intake and BP reliable and robust. Additionally, secondary analyses on the association of UNaV with BP after exclusion of inadequately collected 24h urine samples still showed a highly significant and even stronger positive association between sodium intake and BP, despite the reduction of sample size.

Our current study adds to the rationale for dietary sodium intervention in RTR. The Multiple Risk Factor Intervention Trial (MRFIT) showed that even small differences in population average BP relate importantly to CVD and all-cause mortality. It was estimated that a 4 mmHg reduction of SBP was associated with meaningful benefit: lower ischemic heart disease and CVD death rates by 8-9% and lower all-cause mortality by 5.8% [39]. The study by Cook et al demonstrated that dietary sodium intervention can indeed improve long term outcome [40]. In line it has been proposed that in RTR even moderate BP lowering would already have favorable effects on cardiovascular disease and mortality [4]. Moreover, recent post-hoc analyses from the REIN [41] and the RENAAL/IDNT study [42], showed that a moderately lower sodium intake is associated with a substantially better long term renal and cardiovascular outcome in non-diabetic and diabetic renal patients respectively. To the best of our knowledge, no sodium intervention studies have been performed in RTR, which is remarkable, also considering the well-recognized beneficial effects of even a modest dietary sodium restriction to 90-100 mmol per day in patients with native kidney disease on blood pressure and proteinuria [37, 43].

The feasibility of persistent reduction of dietary sodium has been questioned, especially when it comes to compliance with strict recommendations in current guidelines for high risk groups, including patients with chronic kidney disease. For these groups a sodium intake of no more than 1,500 mg (~70 mmol/d) is advocated [19], based on the DASH-sodium trial, in which sodium intake was maintained at 65 mmol per day for 6 weeks [44]. Intervention studies from our own group showed that reduction to approximately 100 mmol day is feasible in a regular nephrology outpatient setting at least for the 6-week periods of the study [25, 45]. The recent hard end point data suggest that even a modest

reduction in dietary sodium might already be highly beneficial in renal patients [41, 42], which increases the feasibility of obtaining health benefits by dietary sodium restriction. Our current data support the relevance of such an approach in RTR.

Previous epidemiological studies in RTR did not detect an association between sodium intake and BP. However, these studies were either small and therefore likely to be underpowered (9-11), or there was a large time lag between assessment of sodium intake and BP, without BP measurements being performed in a standard way (12). Taken together, these factors have likely hampered the power of those studies to detect an association between sodium intake and BP.

Our cross-sectional data make it reasonable to postulate that BP in RTR is sodium sensitive. This could be related to renal function and/or proteinuria as both renal function impairment and proteinuria are associated with increased sodium sensitivity of BP in native kidneys [45, 46]. Other factors could be the use of drugs interfering in the renin-angiotensin-aldosterone system, that generally render BP sodium-sensitive [47] or the fact that many RTR are overweight, leading to increased sodium sensitivity of BP [48, 49]. Another factor that could underlie our findings is the use of calcineurine inhibitors by the majority of RTR, which has been shown to be related to hypertension [50]. Cyclosporine in particular activates the sympathetic nervous system, upregulates endothelin, inhibits nitric oxide and enhances sodium retention, all of which cause potent vasoconstriction and systemic hypertension [51-53].

Like in the general population, the unfavorable effects of sodium intake on BP in RTR are most likely the consequence of expansion of the extra cellular volume (ECV) and the subsequent rise in cardiac output (CO), a phenomenon that has already been shown to be related to high salt intake in healthy subjects [54, 55]. As result of increased vascular stiffness in RTR, especially in those who underwent pre-transplant dialysis [56], the concomitant compensatory decrease in peripheral vascular resistance fails to occur and hypertension is more likely to develop as compared to healthy subjects.

Notwithstanding the above, the exact mechanisms of interaction of sodium with BP in RTR are incompletely known, mainly because of a lack of sodium intervention studies in RTR.

Conclusion

Dietary sodium intake in RTR is below the level of healthy controls, but well above recommended targets. Our cross-sectional data show a positive association between sodium intake and BP in RTR. These data support the need for sodium intervention studies in RTR to improve blood pressure and cardiovascular and renal risk in this population.

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Dietary Protein, Blood Pressure and Renal Function in Renal Transplant Recipients

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Abstract

Background Hypertension is highly prevalent among renal transplant recipients (RTR) and a risk factor for graft failure and cardiovascular events. Protein intake has been claimed to affect blood pressure (BP) in the general population and may affect renal function. We examined the association of dietary protein with BP and renal function in RTR.

Methods We included 625 RTR (53±13 yrs; 57% male). Protein intake was assessed with a food frequency questionnaire (FFQ), differentiating between animal and plant protein. BP was measured according to strict protocol. Creatinine clearance and albuminuria were measured as renal parameters.

Results Protein intake was 83±12 g/d of which 63% derived from animal sources. BP was 136±17 mmHg systolic and 83±11 mmHg diastolic. Creatinine clearance was 66±26 ml/min; albuminuria 41 [10-178] mg/24h. An inverse, though statistically insignificant, association was found between total protein intake and both SBP (β=-2.22 mmHg per SD, p=0.07) and DBP (β=-0.48 mmHg per SD, p=0.5). Protein intake was not associated with creatinine clearance. Although albuminuria was slightly higher in the highest tertile of animal protein intake compared to the lowest tertile (66 mg/d vs 33 mg/d resp., p=0.03), linear regression analyses did not reveal significant associations between dietary protein and albuminuria.

Conclusion Protein intake exceeded current recommendations. Nevertheless, within the range of protein intake in our RTR population, we found no evidence for an association of dietary protein with BP and renal function. Intervention studies focusing on different protein types are warranted to clarify their effect on BP and renal function in RTR.

Introduction

High blood pressure (BP) is a serious health problem after renal transplantation [1-3]. It is an important risk factor for graft failure, cardiovascular events and mortality in renal transplant recipients (RTR) [3, 4], and usually requires multiple anti-hypertensives to ensure adequate BP control. Remarkably, the mechanisms and treatment of high BP in RTR are poorly defined and management is largely derived from data in non-transplant populations. Better elucidation of the mechanisms underlying high BP in RTR is urgently needed, as emphasized recently [5]. Data in non-transplant populations consistently demonstrate an important role of diet and lifestyle in BP. Well-established dietary factors that favorably affect BP in the general population are weight reduction, reduced salt intake, moderation of alcohol intake and increased potassium intake [6] and in non-transplant renal patients dietary salt restriction [7, 8]. In a first study on BP and dietary factors in RTR we recently reported a positive association between sodium intake and BP [9], suggesting that modification of dietary factors can beneficially influence BP in addition to pharmacological BP regimens. Currently, interest is growing in the influence of dietary patterns and macronutrient intake, including protein, on BP. Dietary protein has also been claimed to affect BP, but the large body of literature on dietary protein and BP in the general population [10-16] is not consistent. In renal patients, dietary protein can affect renal hemodynamics as well as renal protein loss, hence modifying the course of long term renal damage (7-10). By these mechanisms, dietary protein might also affect BP. Concern exists that high protein intake induces high intraglomerular pressure and concurrent hyperfiltration, eventually leading to kidney damage and subsequent hypertension [17, 18]. Although data from intervention studies applying protein restriction in chronic kidney disease (CKD) were not entirely conclusive [19, 20], dietary recommendations for patients with CKD advocate a protein intake of 0.6–0.8 g/kg/d, to decrease renal workload and help delay progression of kidney failure [21].

Considering the vast body of studies on dietary protein in CKD, surprisingly little data is available on the impact of dietary protein in RTR. Data on dietary habits, and on associations of dietary protein with BP and renal function in RTR are virtually lacking, and consequently, the empirical basis for the few available dietary guidelines regarding protein intake for RTR is virtually absent [22, 23]. Consequently, it remains unclear, for medical practitioners as well as for RTR, what the optimal level and favorable source of dietary protein is in this population [24].

In this study, therefore, we aimed to clarify the relation of protein with BP and renal function in stable RTR. To this purpose, we examined dietary habits, and analyzed whether the intakes of total protein and types of protein (plant and animal) were associated with BP and renal function, on cross-sectional analysis in a Dutch patient-based cohort of 625 RTR with a functioning graft for at least one year.

Methods

Design and study population

We conducted an observational study to perform cross-sectional analyses in a large, single center RTR cohort. We invited all RTR (≥ 18 years) with a functioning graft for at least one year who visited our outpatient clinic between November 2008 and March 2011. RTR were all transplanted in our center, had sufficient knowledge of the Dutch language and had no history of drug or alcohol addiction, as reported in their patient files. RTR were on standard antihypertensive and immunosuppressive therapy. Of 817 initially invited RTR, 707 (87%) signed written informed consent to participate in this study. We excluded all patients with missing dietary data, leaving 625 RTR eligible for analysis. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Institutional Review Board (METc 2008/186). The routine regimen included no specific dietary counselling, except for discouraging excess sodium intake and encouraging losing weight in overweight individuals. Patients with diabetes were counselled as appropriate to adapt their dietary habits to achieve normoglycaemia.

Assessment of protein intake

Dietary intake was assessed using a semi quantitative food frequency questionnaire (FFQ) that inquired about intake of 177 food items during the last month. For each item, the frequency was recorded in times per day, week, or month. The number of servings per frequency was expressed in natural units (for example, slice of bread or apple) or household measures (for example, cup or spoon). The questionnaire was self-administered and filled out at home. At the day of the visit to the outpatient clinic, all FFQs were checked for completeness by a trained researcher and inconsistent answers were verified with the patients. Total energy and nutrient intake per day was calculated using a computerized Dutch Food Composition Table taking seasonal variation into account [25]. Because RTR have only sparsely been subject to nutritional studies thus far, we checked consistency of our data on protein intake in our population by comparing the estimated protein intake with 24 hour urinary urea excretion. Therefore, all participants were carefully instructed to collect a 24 hour urine sample according to a strict protocol. Urinary urea excretion was considered as a marker reflecting dietary total protein intake and was used to calculate protein intake according to the method of Maroni and colleagues taking also proteinuria into account (protein intake (g/d) = (0.18 x urinary urea excretion in mmol per 24 hours) + 15 + urinary protein excretion in g per 24 hours) [26, 27]. In addition, excretion of several urinary components was measured to infer dietary intake of additional dietary nutrients like sodium and potassium.

Outcome measurements

All measurements were performed during a morning visit to the out-patient clinic. Fasting BP (mmHg) was measured according to a strict protocol. Participants were left alone in a room in half-sitting position while systolic BP, diastolic BP and mean arterial pressure (MAP) were measured with a semi-automatic device (Dinamap® 1846, Critikon, Tampa, FL, USA). Measurements were performed every minute for fifteen minutes and values of the last three measurements were averaged.

Blood was drawn after an 8-12h overnight fasting period in the morning after completion of the 24h urine collection. Renal function was assessed by 24h urinary creatinine clearance (ml/min), calculated as time-factored urinary creatinine concentration (mg/min) divided by plasma creatinine concentration (mg/mL). Serum creatinine levels were determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Plasma and urinary concentrations of electrolytes and urea were measured using routine clinical laboratory methods, as were serum cholesterol and HbA1c. Urinary albumin concentration was determined by nephelometry (Dade Behring Diagnostic, Marburg, Germany). Total urinary protein concentration was analyzed using the Biuret reaction (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Proteinuria was defined as urinary protein excretion ≥ 0.5 g/24h.

Collection of risk factor data

Information on patients' health status, medical history and medication use was obtained from patient records. Questionnaires were used to obtain information on smoking behavior and alcohol intake. Participants were classified as current smokers, former smokers, or never smokers. Alcohol intake was assessed based on self-reported number of beverages consumed weekly, converted into grams of ethanol per day and divided into quartiles (no alcohol, 0-10 g/d, 10-30 g/d and >30 g/d). Body weight and height were measured with participants wearing indoor clothing without shoes. Body Mass Index (BMI) was calculated as weight divided by height squared (kg/m^2).

Statistical analyses

Data-analysis was performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). Normality was tested with the Kolmogorov-Smirnov test and skewed data were normalized by logarithmic transformation (i.e. albuminuria and proteinuria). Protein intake (total, plant, animal) was adjusted for total energy intake according to the residual method which is based on an isoenergetic principle [28]. Characteristics of the study population and data on dietary intake were calculated in tertiles of energy adjusted total protein intake. Data in text and tables are presented as mean ± standard deviation (SD), unless stated otherwise.

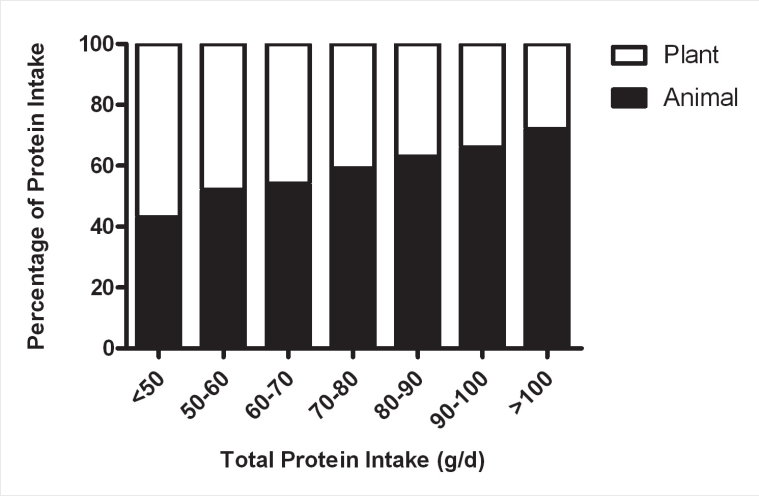
We used multivariable linear regression models to obtain the regression coefficients for BP and renal parameters per SD of energy-adjusted protein intake (total, plant, animal) in RTR. Our basic model (model 1) included age (continuous) and gender. In the second model we further adjusted for BMI (continuous), SBP (continuous; only applied in analyses for the association between protein intake and renal function) smoking behavior (never/ever/current), alcohol consumption (no alcohol, 0-10 g/d, 10-30 g/d and >30 g/d), use of antihypertensive medication (number of drugs; continuous) and time since transplantation (years; continuous). In the final model we additionally adjusted for total energy intake (continuous; kCal/day), urinary sodium, potassium (all continuous; mmol/24h), intake of calcium, magnesium (continuous; mg/d) carbohydrates, saturated fatty acids and polyunsaturated fatty acids (all continuous; g/day).
To allow for non-linear associations, general linear model analyses were used to investigate the associations of tertiles of energy-adjusted protein intake (total, plant, animal) with BP and renal function in RTR. Per tertile of energy adjusted protein intake, the estimated mean values of BP, creatinine clearance and albuminuria were calculated as well as the p-trend across tertiles. Multivariable analyses were repeated with aforementioned adjustments. Within all statistical analyses, a two-sided P value less than 0.05 was considered to indicate statistical significance.

Results

Population characteristics
The mean age of our study population was 53 ± 13 years and 57% was male. Mean BMI was 26.7 ± 4.8 kg/m^2, with 59% of the patients being overweight. Mean BP was 136 ± 17 mmHg systolic and 83 ± 11 mmHg diastolic and 91% of the cohort had hypertension (i.e. BP≥140/90 or use of antihypertensive medication). Of the 625 RTR, 72 (11%) were not using any anti-hypertensive drugs, while 198 (30%) used one antihypertensive drug, 231 (35%) used two and 159 (24%) used three or more different anti-hypertensive drugs. Calcineurin inhibitors (CNIs) were used in 57% of RTR, 2% of RTR used mTOR inhibitors and 83% of RTR was on proliferation inhibiting therapy. Median prednisolone dose was 10 [7.5-10.0] mg/day.
The diet contained 83 ± 12 g/d of energy-adjusted protein (corresponding to 15.5% of an individuals total energy intake (en%) or 1.1 ± 0.3 g/kg/d) of which 52 ± 13 g/d (9.9 en%) derived from animal origin and 31 ± 6 g/d (5.7 en%) from plant origin (mean animal-to-plant ratio approximately 2:1). The distribution of plant protein intake and animal protein intake per group of total protein intake (g/d) is shown in Figure 1. The percentage of protein intake declined from 57% in the lowest group to 28% in the highest

group. Animal-to-plant ratio in the lowest group of total protein intake was 0.75 versus 2.5 in the highest group of total protein intake.

Figure 1 Distribution of plant protein intake and animal protein intake per group of total protein intake (g/d). The percentage of plant protein intake declined from 57% in the lowest group to 28% in the highest group. Animal-to-plant ratio in the lowest group of total protein intake was 0.75 versus 2.5 in the highest group of total protein intake.



Based on the Maroni formula, total protein intake was 85 ± 21 g/d (~1.1±0.3 g/kg/d), which did not significantly differ from the protein estimate derived from the FFQ (p=0.3). Mean intake of calories, calcium, magnesium and phosphorus were 2175 ± 637 kCal/d, 1049 ± 378 mg/d, 331 ± 90 mg/d and 1521 ± 331 mg/d respectively. Mean urinary excretion of sodium and potassium was 157 ± 62 mmol/24h and 73 ± 24 mmol/24h respectively. Of the caloric intake, 36 en% derived from fat (saturated fat 13 en%; monounsaturated fat 12 en%; polyunsaturated fat 8 en%) and 46 en% came from carbohydrates. Mean intake of fiber was 22 ± 7 g/d.

Patient characteristics by tertiles of total energy-adjusted protein intake are shown in Table 1. RTR in the highest tertile of energy adjusted protein intake were likely to be older, to have a higher BMI and higher urinary urea excretion levels, whereas prevalence of males and smokers were lower with higher energy adjusted protein intake. With higher protein intake, RTR tended to increase intake of animal protein rather than that of plant protein, both in absolute (g/d) and relative (en%) amounts.

Table 1 Patient characteristics across tertiles of energy-adjusted total protein intake.

	Tertiles of energy adjusted protein intake (g/d)			
	I n = 208	II n = 209	III n = 208	p
Protein intake, g/d (en%)	71 ± 7 (13.1)	83 ± 3 (15.6)	96 ± 7 (17.8)	
Demographics				
Gender, % male	61	58	51	0.06
Age, y	50 ± 14	54 ± 12	56 ± 12	<0.001
Weight, kg	78 ± 16	79 ± 15	83 ± 17	0.01
Length, cm	174 ± 10	173 ± 10	173 ± 9	0.4
BMI, kg/m^2	25.7 ± 4.6	26.3 ± 4.4	27.7 ± 5.0	<0.001
Current smokers, %	15	14	10	0.27
Time since transplantation, y	6.5 [3.1-12.4]	5.1 [1.6-12.5]	5.1 [1.3-11.9]	0.22
Dietary Intake				
Energy intake, kCal/d	2255 ± 774	2085 ± 564	2185 ± 548	0.024
Animal protein, g/d (en%)	40 ± 9 (7.6)	52 ± 6 (9.9)	65 ± 10 (12.1)	<0.001
Plant protein, g/d (en%)	31 ± 7 (5.6)	31 ± 5 (5.7)	31 ± 6 (5.7)	0.19
Fat, g/d	92 ± 44	84 ± 27	88 ± 29	0.052
Carbohydrate, g/d	269 ± 90	237 ± 71	242 ± 68	<0.001
Calcium intake, mg/d	865± 334	1001± 293	1279 ± 366	<0.001
Magnesium intake, mg/d	316 ± 101	323 ± 84	358 ± 78	<0.001
Fiber intake, g/d	22.2 ± 7.9	22.0 ± 6.6	23.1 ± 5.7	0.21
Alcohol intake[2], g/d	2.0 [0.02-11.6]	3.5 [0.05-13.7]	2.0 [0.02-9.6]	0.06
Medication use				
Antihypertensives, %	86	85	93	0.015
Number of antihypertensives*	2 [1-2]	2 [1-2]	2 [1-3]	0.97
CNI's, n (%)	119 (57)	116 (56)	122 (59)	0.77
mTOR inhibitors, n (%)	6 (3)	1 (0.5)	3 (1)	0.24
Hemodynamic parameters				
Systolic Blood Pressure, mmHg	136 ± 16	137 ± 18	135 ± 18	0.53
Diastolic Blood Pressure, mmHg	83 ± 11	84 ± 11	82 ± 11	0.053
Mean Arterial Pressure, mmHg	100 ± 11	102 ± 13	100 ± 12	0.14
Renal Function Parameters				
Serum Creatinine, umol/l	128 [103-171]	121 [99-155]	126 [99-156]	0.21
Creatinine Clearance, ml/min	63 ± 27	68 ± 25	66 ± 25	0.12
eGFR (ml/min/1.73m^2)	52 ± 21	54 ± 21	56 ± 20	0.26
Urinary Albumin Excretion, mg/24h	36.1 [9.7-176]	38.4 [8.5-149]	50.0 [11.9-202]	0.57
Proteinuria (≥0.5 g/24h), n (%)	45 (22)	48 (23)	45 (22)	0.98

Serum Parameters				
Urea, mmol/l	9.1 [6.9-13.5]	9.0 [7.0-12.7]	10.1 [8.1-13.9]	0.09
Cholesterol, mmol/l	4.9 [4.3-5.7]	5.1 [4.4-5.8]	5.1 [4.4-5.8]	0.47
HbA1c, %	5.8 ± 0.7	6.0 ± 5.8	6.2 ± 0.9	<0.001
Uric acid, mmol/l	0.44 ± 0.12	0.43 ± 0.11	0.44 ± 0.12	0.84
Urinary excretions, mmol/24h				
Phosphate	23.4 ± 8.6	25.6 ± 8.7	26.2 ± 8.9	<0.001
Urea	344 ± 97	397 ± 111	430 ± 119	<0.001
Sodium	142 ± 54	167 ± 66	161 ± 61	<0.001
Potassium	67.4 ± 23.4	74.3 ± 24.7	77.8 ± 24.4	<0.001
Creatinine	11.4 ± 3.6	11.7 ± 3.2	11.7 ± 3.3	0.29
Net Acid Excretion	41.3 ± 20.0	46.7 ± 20.2	48.1± 22.3	0.003

Data are presented as mean ± SD, % or median [IQR]. Abbreviations: en%, energy percentage; BMI, body mass index; CNI, calcineurin inhibitor; mTOR, mammalian target of rapamycin; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin. * among users

Protein intake and BP

Intake of energy-adjusted protein (total, animal, plant) tended to be inversely associated with BP, although the level of significance was not reached (Table 2). After adjustment for potential confounders, the regression coefficients for systolic BP were -2.22 (p=0.07), -1.07 (p=0.37) and -1.41 (p=0.19) per SD of total, animal and plant protein respectively. On secondary regression analyses with tertiles of energy adjusted protein intake (total, animal, plant) instead of with continuous values of protein intake, in that way forcing more contrast in protein intake, findings remained essentially similar. Median total protein intake in the lowest tertile was 73.0 g/d compared to 94.0 g/d in the highest tertile. Although SBP was 3.9 mmHg lower in the highest tertile of protein intake (133.8 mmHg vs 137.7 mmHg in the lowest tertile), this difference did not reach statistical significance (p=0.2). Similar trends were found for the associations of animal and plant protein with BP (data not shown).

Table 2 Regression coefficients of the association between energy adjusted total protein intake and blood pressure in RTR.

Exposure variable	SD	Model	SBP (mmHg)		DBP (mmHg)		MAP (mmHg)	
			β/SD	p	β/SD	p	β/SD	p
Total protein (g/d)	12.0 g/d	1	-0.39	0.59	-0.21	0.63	-0.27	0.59
		2	-0.84	0.25	-0.40	0.39	-0.54	0.28
		3	-2.22	0.07	-0.48	0.54	-1.06	0.22
Animal protein (g/d)	13.1 g/d	1	0.23	0.74	-0.13	0.77	-0.01	0.98
		2	-0.22	0.76	-0.34	0.46	-0.29	0.55
		3	-1.07	0.37	-0.47	0.53	-0.67	0.41
Vegetable protein (g/d)	5.8 g/d	1	-1.22	0.08	-0.12	0.78	-0.49	0.31
		2	-1.14	0.10	-0.05	0.92	-0.41	0.40
		3	-1.41	0.19	0.14	0.83	-0.37	0.62

Model 1: adjusted for age and gender; Model 2: additionally adjusted for BMI, smoking behavior, alcohol intake, antihypertensive drugs and time since transplantation; Model 3: additionally adjusted for total energy intake, urinary sodium and potassium excretion, intake of calcium, magnesium, carbohydrates, saturated fatty acids and polyunsaturated fatty acids. Abbreviations: SD, standard deviation; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

Protein intake and renal function

Table 3 shows the regression coefficients for the association between energy-adjusted protein intake (total, animal, plant) and renal function, reflected by creatinine clearance, albuminuria and proteinuria. Total protein intake was significantly associated with creatinine clearance, independent of age and gender (β=2.17 per SD protein intake; p=0.05). However, the fully adjusted models for the association between protein intake and creatinine clearance yielded insignificant regression coefficients of 0.19 ml/min (p=0.9), 0.17 ml/min (p=0.9) and 0.03 ml/min (p=0.9) per SD of total, animal and plant protein respectively. Also, protein intake was not associated with albuminuria or proteinuria in this RTR cohort, regardless of both protein type and the adjustments that were made (Table 3). In order to consider the effect of CNIs, we also performed an adjustment for CNI use in linear regression analyses which did not essentially change the findings in the complete cohort (data not shown).

Table 3 Regression coefficients of the association between energy adjusted total protein intake and renal function parameters in RTR.

Exposure variable	SD	Model	Creatinine clearance (ml/min)		Albuminuria (log mg/24h)		Proteinuria (log g/24h)	
			β/SD	p	β/SD	p	β/SD	p
Total protein (g/d)	12.0 g/d	1	2.17	0.05	0.07	0.37	0.01	0.97
		2	2.00	0.07	0.09	0.27	0.02	0.77
		3	0.19	0.90	0.12	0.30	0.01	0.91
Animal protein (g/d)	13.1 g/d	1	1.59	0.14	0.06	0.50	0.01	0.86
		2	1.30	0.23	0.05	0.56	0.01	0.95
		3	0.17	0.91	0.08	0.47	0.01	0.99
Vegetable protein (g/d)	5.8 g/d	1	0.73	0.48	0.02	0.77	0.04	0.61
		2	1.05	0.32	0.07	0.35	0.02	0.87
		3	0.03	0.99	0.05	0.67	0.02	0.84

Model 1: adjusted for age and gender; Model 2: additionally adjusted for BMI, SBP, smoking behavior, alcohol intake, antihypertensive drugs and time since transplantation; Model 3: additionally adjusted for total energy intake, urinary sodium and potassium excretion, intake of calcium, magnesium, carbohydrates, saturated fatty acids and polyunsaturated fatty acids. Abbreviations: SD, standard deviation;

With tertiles of energy-adjusted protein intake (total, animal, plant) rather than continuous variables, results were essentially similar; creatinine clearance was 62.4 ml/min in the lowest tertile of energy-adjusted protein intake (median intake 73.0 g/d) versus 66.3 ml/min in de highest tertile of energy-adjusted protein intake (median intake 94.0 g/d; p-trend=0.2). Also, differentiation between animal and plant protein did not alter previous findings (data not shown). With albuminuria, we did not observe significant differences across increasing tertiles of total protein intake (p = 0.15). However, with respect to the intake of energy adjusted animal protein, a significant trend was found. The highest tertile, with a median protein intake of 63 g/d had 66 mg/24h albuminuria, compared with 33 mg/24h albuminuria in the lowest tertile with a median animal protein intake of 41 g/d (p-trend = 0.03). This was independent of age, gender, BMI, SBP, smoking behavior, alcohol intake, anti hypertensive drugs, time since transplantation and dietary factors. These differences in albuminuria were not seen across tertiles of plant protein intake.

Discussion

To date, no evidence is available regarding the optimal level of protein intake and its favorable source (i.e. animal or plant protein) in stable RTR. Therefore, we examined dietary habits in RTR, with the main purpose to study whether dietary protein was associated with BP and renal function parameters in a large, single center RTR cohort. In our analyses among 625 RTR with a functioning graft for at least one year, average protein intake was 83 ± 12 g/d (~1.1 ± 0.3 g/kg/d), thus exceeding recommended values for RTR. Intake of other relevant dietary factors, i.e sodium, phosphorus, fiber and intake and composition of fat was not in compliance with dietary recommendations either. Dietary protein (total, plant, animal) was not associated with BP or creatinine clearance. Although an adverse renal effect of animal protein intake was suggested by a higher albuminuria in the highest tertile of animal protein intake compared to the lowest tertile, no continuous relation was found in linear regression analysis.

This is the first study providing detailed information on dietary habits in RTR. Several methodological aspects of the nutritional assessment deserve to be addressed. First, estimation of animal and plant protein intake was assessed by FFQs, based on self-report, which may have led to misclassification due to inadequacies in dietary recall. However, estimations of total protein intake based on FFQ were similar to estimations based on urinary urea excretion. We therefore do not expect much bias from misclassification regarding animal and plant protein either. Additionally, during the study, a dietary diary was kept for three consecutive days in a subgroup of 60 RTR and dietary data of both

FFQ and diaries were compared. Pearson correlations between FFQ and diaries were 0.72 for energy intake, 0.64 for protein intake, 0.50 for fat intake and 0.69 for carbohydrate intake. These correlation coefficients were comparable with those observed in previous studies analyzing the validity of FFQs in population-based cohort studies [29]. Second, our analyses are based on cross-sectional data with protein intake and BP being measured at the same moment. This makes it difficult to assess the temporal relationships in a potential association. For instance, patients with renal function decline might restrict their protein intake which might have manipulated potential associations. This is, however, unlikely, because no active intervention on protein intake is advocated in RTR when renal function decreases, until dialysis is re-started, to prevent induction of protein-malnutrition in the face of continued immunosuppression. Third, it might be hypothesized that, as a result of heterogeneity of the RTR population (e.g. pharmacological regimens, diversity in allograft vintage), significant associations of dietary protein with BP might go unnoticed. However, despite possible blurring of potential associations due to these factors, classical factors associated with BP in the general population like age, gender, BMI and sodium intake were significantly positively associated with BP in our RTR population [9] thus supporting the power of our study to identify determinants of blood pressure in the current clinical context. Strengths of our study include the fact that, to our knowledge, this is the first study examining the association of dietary protein with BP and renal function in a large, stable RTR population, with the obvious limitation however, of its single-center nature that limits its generalizability. Extensive data collection made it possible to adjust for many potential confounders, including sodium intake reflected by urinary sodium excretion. Previous studies, in line with ours, have shown a firm and inextricable association between protein intake and sodium intake cross-sectionally [8, 30] which therefore makes it difficult to distinguish between the effects of the separate dietary components on BP and renal function.

Our dietary inventory allows a detailed assessment of the dietary habits of the RTR population, albeit in a single center set-up. The dietary habits of our RTR generally are not quite optimal, as shown from their intake of macronutrients as well as sodium and phosphorus, which deviate from the available recommendations. Accordingly, dietary habits can be considered logical targets for intervention in RTR, but this renders it all the more important to reinforce the empirical basis in this population.

Protein intake and BP

No significant associations of dietary protein with BP in RTR were seen, which suggests that dietary protein, within the range of intake in our population, is well tolerated in stable RTR. However, it might be hypothesized that the absence of a significant association is explained by the relatively small range of protein intake in our population. The SD

of unadjusted mean total protein intake in RTR was 20 g/d, which is smaller than the SD of 27 g/d in a big sample of the Dutch general population [31]. RTR usually have a history of long term exposure to strict dietary restrictions, especially during the dialysis period, this may have modulated the eating habits of this specific population to fairly homogeneous pattern, which could mask a potential association of protein intake with BP. Nevertheless, repeating our multivariate analyses in tertiles instead of per SD, forcing more contrast in exposure, did not reveal significant associations either. Future studies could include RTR from different populations and countries to acquire a larger variation in protein intake or intervene on protein intake, by isocaloric exchange for other macro-nutrients.

Protein intake and renal function

The potentially deleterious effect of dietary protein on renal function, suggested by several previous studies [17, 18, 32-34], is ascribed to the induction of high intraglomerular pressure and concurrent hyperfiltration. This adverse phenomenon of dietary protein was not so pronounced in our study in stable RTR, as appears from the non-significant regression coefficients resulting from our statistical analyses. We did not see higher creatinine clearance or higher albuminuria or proteinuria in RTR with a higher total protein intake. However, a slightly, but significantly, higher albuminuria was seen in the highest tertile of animal protein, independent of a.o. age, gender, BMI, BP and dietary factors like energy and sodium intake. This significant association was not seen in tertiles of plant protein, suggesting that it is not protein per se that could influence albuminuria, but that differences exist between types of protein. One other study addressing the association between protein intake and renal function in RTR was performed by Bernardi et al. They studied the role of long-term dietary protein restriction on renal graft function in 42 post-transplant patients with signs of chronic rejection [33]. Patients with moderate protein intake (0.73 ± 0.11 g/kg) maintained unchanged renal graft function, whereas patients with a high protein diet (1.4 ± 0.23 g/kg) ended up with a significantly lower graft function. However, during enrolment all patients received similar dietary recommendations and compliance with protein restriction was not pre-specified but based on urinary urea excretion. At the end of the study patients were compared in two groups stratified by compliance status. Moreover, the low protein diet was provided in combination with a low sodium and low lipid diet which makes it complicated to isolate the effects of moderate protein intake on renal function.

In conclusion, we found no clear-cut association of dietary protein with BP or creatinine clearance within the ranges of protein intake in our population consisting of 625 stable RTR. Although RTR in the highest tertile of animal protein intake had a higher urinary

albumin excretion compared to RTR in the lowest tertile, no continuous association was found between animal protein intake and albuminuria. In general, dietary habits in our RTR deviated from the available guidelines, with intake of protein, saturated fat, sodium and phosphorus being higher, and intake of polyunsaturated fat, carbohydrates and fiber being lower than recommended. These data prompt for further studies addressing the role of dietary factors in the cardiovascular and renal risk in RTR, including the effects of intervention studies.

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4

Dietary Acid Load and Metabolic Acidosis in Renal Transplant Recipients

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Abstract

Background Acidosis is highly prevalent among renal transplant recipients (RTR) and adversely affects cardio-metabolic processes. Well-known factors contributing to acidosis are graft dysfunction and immunosuppressive drugs. Little is known about the potential influence of diet on acidosis in RTR. We examined the association of metabolic acid load with acidosis and with cardiovascular risk factors in RTR. Second we aimed to identify dietary factors associated with acidosis.

Methods We included 707 RTR. Metabolic acid load was assessed by measuring 24h urinary Net Acid Excretion (NAE; i.e. titratable acid + ammonium – bicarbonate). Acidosis was defined as serum [HCO_3^-] < 24 mmol/L. Blood pressure and insulin resistance, reflected by HbA1c, were among cardiovascular risk factors. Diet was assessed with food frequency questionnaires. Linear regression analysis was applied to investigate the association between NAE and acidosis and between dietary factors and acidosis.

Results Mean age was 53±13y, 57% was male. Acidosis was present in 31% of RTR. NAE was associated with acidosis (serum HCO_3^-: β=-0.61 and serum pH: β=-0.010; both $p<0.001$). Patients with high intake of (animal) protein (i.e. from meat, cheese and fish) and low intake of fruits and vegetables had significantly lower serum HCO_3^- and serum pH. No associations were observed between NAE and cardiovascular risk factors like hypertension and insulin resistance.

Conclusion In addition to conventional factors contributing to acidosis, diet might influence acid-base homeostasis in RTR. Higher intake of fruits and vegetables and lower animal protein intake is associated with less acidosis in RTR.

Introduction

Worldwide, the prevalence of end stage renal disease (ESRD) is increasing rapidly [1]. Although, kidney transplantation is the preferred treatment for patients with ESRD, successful transplantation is still associated with substantially elevated morbidity and mortality [2, 3]. Many renal transplant recipients (RTR) have metabolic acidosis [4, 5] which may adversely affect cardio-metabolic processes, including blood pressure (BP) and insulin resistance as well as proper functioning of multiple tissues [4, 6, 7]. Therefore, it is important to identify modifiable determinants of metabolic acidosis that might help improve acid-base homeostasis in RTR.

Diet can influence acid-base balance in humans [8-10]. Potassium salts of metabolizable anions, like citrate, have an alkalinizing effect, whereas acid load originates from precursors like cationic amino acids and organic acids. Accordingly, fruits and vegetables contribute to base load whereas (animal) protein adds to acid load. The contemporary Western diet, including large amounts of animal products, generates an acid load that is not compensated for by the shortage of fruit and vegetable, subsequently inducing increasing but unnoticed metabolic acidosis [11].

The kidney plays an important role in acid-base homeostasis by excreting the excess of acids ingested. In general, persons consuming diets high in acid load have higher urinary acid excretion compared to persons eating diets rich in alkalinizing foods [12]. In RTR however, the capacity to excrete acid is decreased due to impaired renal function. Therefore, it is hypothesized that RTR could benefit from dietary modifications, to improve acid-base homeostasis. Although post-transplant acidosis has been studied before [4, 5], to our knowledge no studies are available on the role of nutrition in acidosis in RTR.

We therefore examined whether NAE, reflecting metabolic acid load, was related to acidosis in a large RTR cohort. Second, we studied the association of NAE with cardiovascular risk factors like insulin resistance and high BP. Additionally, we aimed to identify dietary factors contributing to NAE in RTR, to provide tools for lowering acidosis in RTR.

Methods

Design and study population

We conducted cross-sectional analyses in a large, single-centre RTR cohort. We invited all adult RTR (age ≥ 18 years) with a functioning graft for at least one year who visited our outpatient clinic between 2008 and 2011. RTR were all transplanted in our center, had sufficient knowledge of the Dutch language and had no history of drug or alcohol addiction, as reported in their patient files. Patients with overt congestive heart failure and patients diagnosed with cancer other than cured skin cancer were not considered eligible for the study. In patients with fever or other signs of infection (e.g. complaints of urinary or upper respiratory tract infection), visits were postponed until symptoms had resolved. Of 817 initially invited RTR, 707 (87%) signed informed consent to participate. All but three were Caucasian. For analyses regarding dietary intake, we excluded all patients with missing dietary data, resulting in 625 RTR for analyses. RTR with complete data and RTR with missing data were similar regarding age, gender, body composition, eGFR and medication use. Of RTR that did not consent, we only recorded age, gender, body composition and eGFR. Compared to participating RTR, RTR that did not consent were slightly older (58±13 vs 53±13 years in both other groups) and had lower eGFR (47±19 vs 51±21 and 53±20 ml/min/1.73m^2 for the RTR with missing and complete data respectively). The Institutional Review Board approved the study protocol (METc 2008/186), which was in adherence to the Declaration of Helsinki.

Assessment of metabolic acid load

Participants were instructed to collect a 24 hour urine sample according to strict protocol at the day prior to their visit to the outpatient clinic. Urine was collected under oil and chlorhexidin was added as antiseptic agent. After completion, electrolytes (chloride, potassium, sodium, calcium and phosphate) were directly analyzed following standard laboratory procedures. Urine pH and titratable acid (TA) were measured with an automated titrator (Metrohm, 855 Robotic Titrosampler, Switzerland). Directly after collection, additional urine samples were stored at -80° Celsius and kept deeply refrigerated for a maximum of two years. Ammonium (NH_4^+) and bicarbonate (HCO_3^-) were measured chromatographically in freshly thawed 24h urine samples (Waters, Alliance HT 2795 and Metrohm, type 861, Herisau, Switzerland respectively). Stability of both parameters over repeated freeze-thaw cycles was analyzed in advance of the study, rendering insignificant differences after thawing compared to the original mean values. NAE, the gold standard for measuring metabolic acid load, was calculated in the conventional manner as TA + NH_4^+ - HCO_3^- [13].

Assessment of diet and acid load

Diet was assessed using a semi quantitative food frequency questionnaire (FFQ) inquiring about intake of 177 items during the last month taking seasonal variations into account. The FFQ was developed at Wageningen University [14] and has been updated several times. For the present study, the FFQ was slightly modified for accurate assessment of protein intake, including types and sources of protein. For each item, the frequency was recorded in times per day, week, or month. The number of servings was expressed in natural units (e.g. slice of bread or apple) or household measures (e.g. cup or spoon). The questionnaire was self-administered and filled out at home. All FFQs were checked for completeness by a trained researcher and inconsistent answers were verified with the patients. Dietary data were converted into daily nutrient intake using the Dutch Food Composition Table of 2006 [15]. The validity of the FFQ in RTR was checked by comparing the estimated protein intake with calculations of protein intake according to the Maroni formula, based on 24h urinary urea excretion, reliably reflecting protein intake [16, 17]. Based on this formula, protein intake was 85±21 g/d (~1.1±0.3 g/kg/d), similar to the estimates derived from the FFQ (83±20 g/d (~1.1±0.3 g/kg/d; p=0.3). Also, in a subgroup of 60 RTR, we compared dietary data of FFQ with three-day food records. Pearson correlations between FFQ and records were 0.72 for energy intake, 0.64 for protein intake (0.53 for animal protein; 0.73 for plant protein), 0.50 for fat intake and 0.69 for carbohydrate intake, comparable with those observed in previous studies analyzing validity of FFQs in population-based cohort studies [18]. Regarding fruit and vegetable intake, correlation coefficients were 0.66 and 0.41 respectively.

Dietary acid load was assessed with two validated methods. First, potential renal acid load (PRAL) was calculated using the algorithm described by Remer et al [10]: PRAL (mEq/d) = 0.4888 x protein intake (g/d) + 0.0366 x Phosphorus (mg/d)–0.0205 x Potassium (mg/d) – 0.0125 x Calcium (mg/d) – 0.0263 x Magnesium (mg/d). Second, we estimated dietary acid load using the algorithm described by Frassetto et al [9]: estimated net endogenous acid production (NEAP; mEq/d) = [54.5 x Protein intake (g/d) / Potassium intake (mEq/d)] – 10.2.

Outcome measures

All measurements were performed after an 8-12h overnight fasting period. BP (mmHg) was measured semi-automatically according to strict protocol as described previously [19]. Measurements were performed every minute for fifteen minutes and the last three measurements were averaged. Renal function was assessed by estimating glomerular filtration rate (eGFR) applying the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [20]. Serum creatinine was determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Blood was

drawn in the morning after completion of the 24h urine collection. Concentrations of electrolytes and urea were measured using routine clinical laboratory methods, as were serum cholesterol, HbA1c and Nt-Pro-BNP-levels. Venous blood gas analyses were assessed photometrically. Acidosis was defined as serum [HCO_3^-] < 24mmol/l.
Information on medical history and medication use was obtained from patient records. Data on smoking behavior (current, former, never) was obtained with an additional questionnaire. BMI was calculated as weight divided by height squared (kg/m^2) and BSA was estimated applying the universally adopted formula of DuBois [21].

Statistical analyses
Data-analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, USA). Skewed data were log-transformed for analyses (i.e. albuminuria, proteinuria, PTH, Nt-pro-BNP, triglycerides, cholesterol). Data are presented as mean ± standard deviation (SD), unless stated otherwise. Patient characteristics were calculated across gender stratified tertiles of NAE, controlled for BSA. P for trend was obtained by using medians as continuous variables in univariate linear regression analysis. For nominal and ordinal variables we applied the χ^2-test and Jonckheere-Terpstra-test respectively.
Linear regression was applied for the associations of NAE with acidosis and cardiovascular risk factors. Regression coefficients are given as standardized βs, referring to the number of standard deviations the dependent variable changes, per standard deviation increase of NAE. Adjustments were made for age, BSA and gender (model 1), use of medication (proliferation inhibitors, calcineurin inhibitors, RAS-inhibitors, sodium bicarbonate and diuretics (model 2) and eGFR (ml/min/1.73m^2), time since transplantation (years) and smoking behavior (categories; model 3). To separate effects of low eGFR and metabolic acid load on acidosis in RTR, we performed regression analyses for 1) the association between eGFR and acidosis and 2) the association between NAE and acidosis.
To identify dietary factors contributing to NAE, we calculated dietary intake across tertiles of NAE, controlling for BSA. Then we applied linear regression analyses, adjusting for age and gender (model 1) eGFR, time since transplantation and smoking behavior (model 2). Regression coefficients are given as change of NAE per SD increase of the dietary factor. A two-sided P value less than 0.05 was considered statistically significant.

Results

Patient characteristics

Mean age was 53±13 years, 57% was male. BMI was 26.7 ± 4.8 kg/m^2, with 59.4% of RTR being overweight (i.e. BMI≥25 kg/m^2). Mean BP was 136±17 mmHg systolic and 83±11 mmHg diastolic; 91% of RTR had hypertension (i.e. BP≥140/90 or use of antihypertensive medication). Of 707 RTR, 637 (89%) used anti-hypertensive drugs; 198 (30%) used one, 231 (35%) used two, and 159 (24%) used three or more different anti-hypertensive drugs. Calcineurin and proliferation inhibitors were used in 57% and 83% of RTR respectively. Median prednisolone dose [interquartile range] was 10 [7.5-10.0] mg/day. Only 3 RTR (0.4%) used sodium bicarbonate. Acidosis (i.e. serum HCO_3^- < 24mmol/l) was present in 31% of RTR and about half of these patients had a systemic pH < 7.35 (figure 1). RTR with acidosis had a significantly lower eGFR compared to non-acidotic RTR (44±20 ml/min/1.73m^2 versus 56±19 ml/min/1.73m^2 respectively; p<0.001).

Mean NAE was 40.7±18.1 mEq/d, ranging from 22.2±10.2 to 60.0±11.8 mEq/d across tertiles of NAE (table 1). NAE was positively associated with eGFR, use of mycophenolate, serum chloride and urinary excretion of ammonia, TA, phosphorus and sulphate, whereas NAE was inversely related to time since transplantation, use of azathioprine, serum and urine pH, and serum and urine bicarbonate. No significant differences were found regarding smoking behavior over tertiles of NAE.

Figure 1 Histogram of serum bicarbonate concentration in a single-center RTR cohort including 707 RTR with a functioning graft for at least one year. Metabolic acidosis (i.e. [HCO_3^-] < 24mmol/l) was present in 31% of RTR.

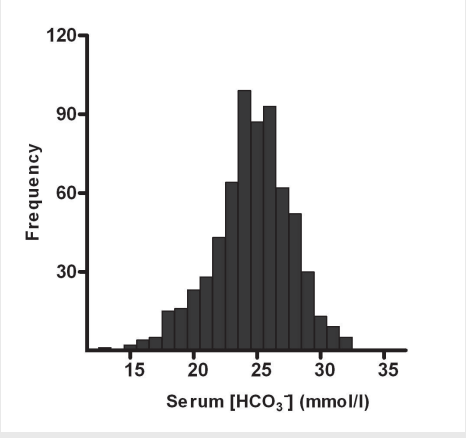

Table 1 Characteristics of 707 renal transplant recipients (RTR) across gender stratified tertiles of urinary net acid excretion (NAE; mEq/24h) controlled for body surface area (BSA)

	Gender stratified tertiles of Net Acid Excretion/BSA*1.73 (mEq/d)			p-trend*
	1 n=236	2 n=235	3 n=236	
NAE *(mEq)*	22.2 ± 10.2	39.8 ± 5.3	60.0 ± 11.8	
Demographics				
Age, years	54 ± 13	53 ± 13	52 ± 12	0.07
BSA, m^2	1.94 ± 0.22	1.95 ± 0.22	1.93 ± 0.22	0.62
Current smokers, %	11	13	14	0.43
Diabetes Mellitus**, %	27	22	23	0.34
Hypertensives, %	91	92	89	0.50
Systolic BP, mmHg	136 ± 18	137 ± 17	136 ± 17	0.89
Diastolic BP, mmHg	83 ± 11	84 ± 10	83 ± 11	0.99
Renal parameters				
Time since Rtx, y	7.7 [3.2-15.2]	5.8 [2.0-11.9]	4.3 [1.3-10.0]	<0.001
Serum Creatinine, umol/L	139 [107-176]	121 [98-153]	116 [97-152]	0.006
eGFR, ml/min/1.73 m^2	48 ± 20	54 ± 20	56 ± 20	<0.001
Albumin excretion, mg/24h	58 [11-202]	35 [9-200]	36 [12-124]	0.26
Proteinuria (≥0.5 g/24h), %	23	25	20	0.52
Medication users				
Sodium bicarbonate, n	1	2	0	0.9
Antihypertensive drugs, %	88	90	86	0.65
Proliferation inhibitor, %	77	84	90	0.001
Mycophenolate, %	47	71	80	<0.001
Azathioprine, %	30	12	10	<0.001
Calcineurin inhibitor, %	57	58	57	0.88
Ciclosporin, %	37	44	37	0.95
Tacrolimus, %	17	16	20	0.53
Diuretics, %	49	36	37	0.01
Anti-diabetic drugs, %	15	16	14	0.66
Serum parameters				
pH	7.38 ± 0.04	7.37 ± 0.05	7.36 ± 0.04	0.001
HCO_3^-, mmol/L	25.1 ± 3.0	24.7 ± 3.0	24.1 ± 3.2	0.004
v-pCO2, kPa	5.85 ± 0.80	5.86 ± 0.83	5.82 ± 0.82	0.71
Base excess	0.5 [-1.3-1.9]	0.2 [-1.9-1.5]	-0.5 [-2.9-1.1]	0.001
Anion gap, mmol/L	11.1 ± 2.2	10.8 ± 2.1	10.7 ± 2.1	0.25

Chloride, mmol/L	104.5 ± 3.4	105.3 ± 3.2	106.2 ± 3.7	<0.001
Phosphate, mmol/L	1.01 ± 0.22	0.97 ± 0.22	0.92 ± 0.19	<0.001
PTH, pmol/L	9.1 [5.9-15.6]	8.5 [5.0-13.9]	9.1 [6.3-14.4]	0.64
Albumin, mmol/L	42.5 ± 3.2	43.2 ± 2.9	43.4 ± 2.8	0.002
Cholesterol, mmol/L	5.30 [4.50-6.00]	5.00 [4.40-5.70]	4.90 [4.20-5.70]	0.04
HDL-cholesterol, mmol/L	1.30 [1.10-1.76]	1.30 [1.10-1.60]	1.30 [1.00-1.60]	0.53
Triglycerides, mmol/L	1.70 [1.29-2.30]	1.63 [1.22-2.34]	1.69 [1.16-2.21]	0.58
HbA1c, %	6.0 ± 0.7	6.0 ± 0.9	6.0 ± 0.8	0.91
hsCRP	1.9 [0.9-6.0]	1.6 [0.7-3.0]	1.4 [0.6-4.7]	0.06
Nt-pro-BNP	340 [143-787]	239 [96-594]	185 [85-442]	0.006
Urinary parameters				
Ammonium, mEq/24h	13.3 ± 7.2	20.0 ± 7.6	32.2 ± 12.3	<0.001
Titratable Acid, mEq/24h	21.0 ± 7.6	30.2 ± 7.9	39.4 ± 11.4	<0.001
HCO_3^-, mEq/24h	7.2 [3.9-12.8]	4.2 [2.3-6.9]	3.3 [2.2-4.9]	<0.001
pH	6.36 ± 0.46	6.00 ± 0.42	5.76 ± 0.45	<0.001
Urea, mmol/24h	341 ± 99	384 ± 101	452 ±119	<0.001
Creatinine, mmol/24h	11.1 ± 7.9	11.7 ± 3.4	12.7 ± 3.4	0.003
Phosphorus, mmol/24h	21.0 ± 7.8	25.1 ± 8.0	29.4 ± 8.5	<0.001
Sulphate, mmol/24h	15.2 ± 5.8	17.6 ± 5.4	20.9 ± 6.9	<0.001
Sodium, mmol/24h	147 ±65	155 ± 58	170 ± 60	<0.001
Potassium, mmol/24h	73.0 ± 25.1	72.9 ± 24.4	73.9 ± 24.0	0.72
Calcium, mmol/24h	2.1 [0.9-3.4]	2.5 [1.2-4.2]	2.5 [1.2-4.2]	0.08

Data are presented as mean ± SD, % or median [IQR]. Abbreviations: NAE, net acid excretion; BSA, body surface area; BP, blood pressure; RTx, renal transplantation; eGFR, estimated glomerular filtration rate; v-pCO_2, venous partial pressure of CO_2; PTH, parathyroid hormone; HbA1c, glycosylated haemoglobin; hsCRP, high-sensitive CRP; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide. * P-trend was tested by either entering the median values within tertiles into the model as covariate, by χ^2-test or by Jonckheere-Terpstra test; **Diabetes Mellitus was defined as serum glucose ≥ 7mmol/L or use of anti-diabetic drugs.

NAE, acidosis and cardiovascular risk profile

The associations between NAE, serum HCO_3^- and various cardiovascular risk factors are shown in Table 2. After adjustment for age, gender, BSA, use of medication, eGFR, time since transplantation and smoking behavior, NAE was inversely associated with serum bicarbonate and serum pH (St. β=-0.16; p<0.001 and St. β=-0.18; p<0.001 respectively). Similar associations were found between PRAL and NEAP and acidosis: adjusted regression coefficients (model 3) for the association of PRAL and NEAP with serum HCO_3^- were -0.13 and -0.12 respectively (both p=0.001). Standardized βs for the

associations of PRAL and NEAP with serum pH were -0.07; p=0.07 and -0.09 p=0.02 respectively). In secondary analyses, the association of NAE with HCO_3^- and serum pH appeared to be significant in RTR with and without metabolic acidosis (all p<0.01; data not shown).

Further analyses revealed standardized betas of 0.33 and 0.31 for the association of eGFR with serum [HCO_3^-] and serum pH respectively (both p<0.001), whereas standardized betas were -0.08 (p=0.005) and -0.10 (p=0.001) for the association of NAE with serum [HCO_3^-] and serum pH respectively. With respect to cardiovascular risk factors, NAE was inversely associated with total cholesterol (St. β=-0.10, p=0.02) and serum phosphate (St. β=-0.12, p=0.003) (model 3). Initially significant associations between NAE and serum albumin (inverse) and serum Nt-Pro-BNP (direct) disappeared after adjustment for eGFR, time since Rtx and smoking behavior (table 2). NAE was not related to BP and insulin resistance (table 2).

Dietary factors and NAE

Both formulas estimating dietary acid load were positively correlated with NAE, i.e. r=0.42 (p<0.001) for the correlation between PRAL and NAE and r=0.32 (p<0.001) for the correlation between NEAP and NAE. Dietary intake across gender stratified tertiles of NAE, controlled for BSA, is shown in table 3. Across tertiles, NAE was positively associated with total protein, animal protein, phosphorus and calcium (all p-trend<0.05). Regarding food groups, NAE was directly related to cheese intake (p-trend=0.001) and inversely related to fruit intake (p-trend=0.05). When analyzing continuously (table 4), NAE was positively associated with intake of total protein (β=2.88; p=0.001), animal protein (β=3.25; p<0.001), calcium (β=2.62; p=0.002) and phosphorus (β=2.51; p=0.004) after adjustment for age and gender. These associations remained essentially unchanged after adjustment for eGFR, time since transplantation and smoking behavior (total protein (β=2.17; p=0.006), animal protein (β=3.08; p<0.001), calcium (β=2.41; p=0.006) and phosphorus (β=2.28; p=0.008)). Translation to food groups showed that those nutrients might originate from meat, fish and cheese since significant associations were observed of NAE with meat (β=1.81; p=0.04); fish (β=1.91; (p=0.01); and cheese (β=3.07; p<0.001). Also, intake of bread was directly associated with NAE (β=2.10; p=0.02), whereas we observed inverse associations between NAE and intake of fruits (β=-1.77; p=0.04) and vegetables (β=-1.79; p=0.05).

Table 2 Regression coefficients for the association of urinary net acid excretion (NAE) with metabolic acidosis and related cardiovascular risk factors in 707 renal transplant recipients

		Model 1				Model 2				Model 3			
Dependent variable	SD	St. β for NAE	95% CI lower	upper	p	St. β for NAE	95% CI lower	upper	p	St. β for NAE	95% CI lower	upper	p
Systemic acidosis													
Serum HCO_3^-, mmol/l	3.01	-0.08	-0.14	-0.02	0.005	-0.17	-0,28	-0,05	<0.001	-0.16	-0.23	-0.10	<0.001
Cardiovascular risk factors													
Serum pH	0.04	-0.10	-0.15	-0.03	0.001	-0.20	-0,30	-0,07	<0.001	-0.18	-0.24	-0.12	<0.001
Systolic BP, mmHg	17	0.009	-0.51	0.46	0.97	-0.2	-2,13	2,52	0.87	-0.01	-0.05	0.03	0.75
Diastolic BP, mmHg	11	0.04	-0.14	0.22	0.66	0.02	-0,04	0,08	0.52	0.02	-0.09	0.13	0.57
MAP, mmHg	12	0.03	-0.21	2.72	0.80	0.004	-0,01	0,02	0.64	0.008	-0.29	0.27	0.84
Cholesterol, mmol/l	1.12	-0.12	-0.19	-0.05	0.002	-0.11	-0,19	-0,03	0.006	-0.10	-0.17	-0.03	0.02
HDL-cholesterol, mmol/l	0.48	-0.02	-0.06	0.02	0.28	-0.05	-0,13	0,03	0.20	-0.03	-0.06	0.00	0.46
LDL-cholesterol, mmol/l	0.93	-0.09	-0.17	-0.02	0.02	-0.08	-0,16	-0,01	0.05	-0.07	-0.14	0.00	0.10
Triglycerides, mmol/l*	0.47	-0.10	-0.19	-0.01	0.03	-0.09	-0,20	0,02	0.12	-0.08	-0.19	0.03	0.06
Phosphate, mmol/l	0.21	-0.20	-0.29	-0.11	<0.001	-0.11	-0,16	-0,06	<0.001	-0.12	-0.21	-0.02	0.003
Albumin, g/l	2.98	0.13	0.03	0.23	0.01	0.07	-0,01	0,14	0.06	0.06	-0.06	0.18	0.13
Nt-Pro-BNP, ng/l*	1.42	-0.16	-0.26	-0.06	0.002	-0.07	-0,13	-0,02	0.01	-0.05	-0.24	0.14	0.17
HbA1c, %*	0.13	0.05	-0.01	0.12	0.15	0.03	0,01	0,06	0.17	0.04	-0.01	0.10	0.32
Albuminuria, mg/24h*	1.92	-0.08	-0.26	0.14	0.39	0.02	-0,13	0,09	0.72	0.01	-0.01	0.02	0.75
Proteinuria, g/24h*	1.72	-0.07	-0.17	0.03	0.52	0.04	-0,13	0,05	0.59	0.02	-0.07	0.11	0.70

Association of NAE as independent variable, adjusted for age (y), BSA (m^2) and gender (model1); RAS-blockade, diuretics, calcineurin inhibitors, proliferation inhibitors and sodium bicarbonate (yes/no) (model 2); and renal function (eGFR), time since transplantation (y) and smoking behavior (model 3). *Log-transformed for analyses. Abbreviations: CI confidence intervals; HCO_3^- bicarbonate; BP blood pressure; MAP mean arterial blood pressure; HDL high-density lipoprotein; LDL low-density lipoprotein; Nt-Pro-BNP N-terminal pro-brain natriuretic peptide; HbA1c, glycosylated haemoglobin. Standardized βs refer to the number of standard deviations the dependent variable changes per standard deviation increase of NAE.

Table 3 Dietary intake of 625 renal transplant recipients across gender stratified tertiles of net acid excretion (NAE; mEq/d) controlled for body surface area (BSA).

	Gender stratified tertiles of Net Acid Excretion/BSA*1.73 (mEq/d)			
	1 n=208	2 n=209	3 n=208	P trend
Nutrients				
Energy intake, kCal/d	2141 ± 607	2252 ± 608	2174 ± 700	0.61
Protein intake, g/d (en%)	79 ± 20 (15.2)	83 ± 19 (14.7)	85 ± 20 (16.1)	0.02
Animal protein, g/d (en%)	50 ± 15 (9.6)	52 ± 15 (9.5)	54 ± 15 (10.4)	0.01
Vegetable protein, g/d (en%)	30 ± 9 (5.6)	32 ± 10 (5.8)	31 ± 10 (5.7)	0.48
Fat, g/d (en%)	87 ± 32 (36.0)	92 ± 31 (36.3)	89 ± 39 (36.6)	0.56
Carbohydrates, g/d (en%)	247 ± 75 (46.5)	259 ± 78 (46.1)	244 ± 80 (44.8)	0.67
Fiber, g/d	22 ± 7	24 ± 7	22 ± 7	0.54
Calcium, mg/d	1009 ± 353	1058 ± 358	1109 ± 420	0.01
Magnesium, mg/d	324 ± 86	345 ± 91	333 ± 91	0.34
Potassium, mg/d	3496 ± 867	3667 ± 912	3499 ± 906	0.34
Phosphorus, mg/d	1477 ± 387	1559 ± 374	1568 ± 420	0.02
Food groups				
Meat, g/d	94 ± 40	97 ± 40	97 ± 40	0.47
Fish, g/d	11.4 [3.0-22.3]	11.7 [4.7-19.4]	10.7 [4.2-20.9]	0.17
Milk, g/d	116 ± 83	120 ± 81	114 ± 89	0.81
Cheese, g/d	32 ± 25	34 ± 26	41 ± 32	0.001
Fruit, g/d	161 ± 122	156 ± 117	140 ± 105	0.05
Vegetables, g/d	95 ± 58	99 ± 63	88 ± 52	0.23
Potatoes, g/d	134 ± 77	135 ± 72	125 ± 80	0.28
Bread, g/d	123 ± 54	145 ± 67	136 ± 58	0.03
Dietary acid load formulas				
PRAL, mEq/d	0.32 [-8.1;7.2]	0.95 [-5.8;6.8]	5.1 [-3.2;11.1]	<0.001
NEAP, mEq/d	39 ± 9	40 ± 8	42 ± 8	<0.001

Data are presented as mean ± SD, % or median [IQR]. Abbreviations: en%, energy percentage; PRAL, potential renal acid load; NEAP, net endogenous acid production. P-trend was tested by either entering the median values within tertiles into the model as covariate, by χ^2-test or by Jonckheere-Terpstra test.

Table 4 Regression coefficients for the association of dietary factors with NAE/BSA*1.73 (mEq/24h) in 625 patients

Dietary factor		Model 1				Model 2			
Nutrients	SD	β/SD	95% CI lower	95% CI upper	p	β/SD	95% CI lower	95% CI upper	p
Energy intake, kCal/d	599	0.50	-1.37	2.38	0.60	0.33	0.03	0.63	0.76
Protein intake, g/d	20	2.88	0.93	4.83	0.001	2.17	0.12	4.20	0.006
Animal protein, g/d	15	3.25	1.21	5.29	<0.001	3.08	0.59	5.59	<0.001
Vegetable protein, g/d	9	0.70	-0.64	2.04	0.43	0.63	-2.12	3.39	0.41
Fat, g/d	32	0.45	-5.04	5.94	0.63	0.27	1.03	-0.49	0.74
Carbohydrate, g/d	73	-0.67	-3.78	2.44	0.45	-0.67	-1.95	0.61	0.57
Fiber, g/d	7	-0.67	-2.34	1.00	0.43	-0.63	-1.71	0.45	0.39
Calcium, mg/d	359	2.62	0.81	4.43	0.002	2.41	0.31	4.51	0.006
Magnesium, mg/d	85	1.36	-0.27	2.99	0.12	1.03	-0.82	2.88	0.16
Potassium, mg/d	872	0.39	-0.45	1.23	0.65	0.14	0.93	-0.65	0.89
Phosphorus, mg/d	390	2.51	0.68	4.34	0.004	2.28	0.03	4.53	0.008
Food groups									
Meat, g/d	40	1.74	0.23	3.27	0.04	1.81	0.44	3.18	0.04
Fish, g/d	16	1.86	0.14	3.58	0.03	1.91	0.46	3.36	0.01
Milk, g/d	85	0.40	-1.16	1.96	0.65	0.65	-3.31	4.61	0.67
Cheese, g/d	28	3.04	1.11	4.97	<0.001	3.07	0.81	5.33	<0.001
Fruit, g/d	115	-1.65	-3.13	-0.17	0.05	-1.77	-3.46	-0.08	0.04
Vegetables, g/d	58	-1.55	-3.05	-0.17	0.04	-1.79	-3.90	-0.16	0.05
Potatoes, g/d	77	-1.15	-2.50	0.20	0.16	-1.21	-2.69	0.27	0.16
Bread, g/d	60	2.15	0.35	3.94	0.008	2.10	0.29	5.33	0.02
Dietary acid load formulas									
PRAL, mEq/d	12	3.37	1.79	4.95	<0.001	3.24	1.49	4.99	<0.001
NEAP, mEq/d	9	3.41	1.86	4.96	<0.001	3.38	1.62	5.14	<0.001

Model 1: adjusted for age (y) and gender; model 2: additionally adjusted for renal function (eGFR), time since transplantation (y) and smoking behavior; Abbreviations: BSA, body surface area; SD, standard deviation; 95% CI, 95% confidence interval; PRAL, potential renal acid load; NEAP, net endogenous acid production.

Discussion

In this study, we examined the relation between diet and NAE (reflecting metabolic acid load). Additionally we studied the association of NAE with acidosis and various cardiovascular risk factors in 707 RTR. Acidosis was present in 31% of RTR. NAE was positively associated with acidosis, whereas no associations were observed with insulin resistance and high BP. NAE was higher in patients with higher intake of (animal) protein, (presumably from cheese, meat and fish) and calcium, and lower in patients with higher intakes of fruits and vegetables.

Strengths of our study include the large sample size and the novelty of our findings in this specific patient group. Furthermore, collection of 24h urine samples allowed direct measurement of NAE as marker of metabolic acid load in addition to estimation of dietary acid load based on dietary recall (PRAL and NEAP). This enabled us to verify internal consistency of our data and made our conclusions more reliable. Limitations are the observational and cross-sectional study design which does not allow for proving causality. However, although reverse causality should be considered, it seems unlikely that RTR with higher NAE would have adapted dietary habits, since the extent of urinary acid excretion goes unnoticed. Second, NAE is a marker of the total amount of acids ingested, and therefore reflects both acidity of the diet and use of acidifying (or alkalinizing) drugs. Nevertheless, both PRAL and NEAP were significantly associated with NAE which seems to validate NAE as marker of dietary acid load. Third, almost all RTR were Caucasian, which calls prudence to extrapolation of our findings to other ethnicities. Furthermore, although extensive data collection allowed controlling for many confounders residual confounding could have remained even in multivariable analysis, since it is hard to adjust completely for the severity of each factor. Therefore, prospective cohort and intervention studies in RTR are needed to confirm our results.
Previously, Ashurst *et al.* showed that CKD patients with persistent low serum bicarbonate levels had a larger annual decline in eGFR compared to patients with normal bicarbonate levels [22]. In another study, they observed that CKD patients supplemented with sodium bicarbonate were less likely to develop ESRD, which is in line with findings of Phisitkul *et al.*, who showed that treatment of metabolic acidosis with sodium citrate in patients with low GFR reduced progression of kidney disease [23, 24]. Interestingly, a recently published study showed that reduction of dietary acid by increasing intake of fruits and vegetables lead to reduced kidney injury in subjects with CKD stage 2, which is in line with our findings in RTR [25].
Acidosis has since long been recognized as risk factor in RTR, and several potential causes have been suggested [26-30]. Well known contributors to acidosis are reduced

nephron mass, resulting in decreased acid excretion, and use of pharmacological agents, like calcineurin inhibitors, that directly influence acid-base status but also play a significant role in renal acid handling. Thus far, no studies have been performed on the contribution of diet to acid-base status in RTR, despite the vast body of evidence that food intake does affect acid-base balance in both the general population and CKD patients [8, 10, 25]. In line, we found protein intake to be directly associated with NAE in RTR. This finding was confirmed by significant associations between urinary urea, phosphorus and sulphate excretion, which all have been suggested reliable markers of protein intake [31, 32]..

Although robust associations between NAE and acidosis in RTR were observed, no direct associations were found between NAE and cardiovascular risk factors, which is in contrast with findings of previous studies [33-36]. Differences in outcomes might be explained by differences in study populations since previous studies were done in healthy subjects without use of medication. Since respectively 16% and 88% of RTR used anti-diabetic and anti-hypertensive drugs, this might have interfered in the potentially existing associations between NAE and cardiovascular risk factors like insulin resistance and hypertension. Another explanation might be that potential adverse effects of acidosis on e.g. BP were not detected in this cross-sectional study because both parameters were measured at the same time. Unfavorable effects of acidosis on the long run might however become visible when prospective data were obtained.

If confirmed by prospective and intervention studies, our findings might have several implications for clinical practice. Since acidosis is highly prevalent among RTR, venous blood gas analysis should be performed occasionally since it provides important information on acid-base status relatively easily. If acidosis is confirmed, attention should be paid not only to known risk factors like graft function, but also to dietary habits. Either higher intake of fruits and vegetables or lower intake of (animal) protein should be advised. Based on our results, daily enrichment of the diet with 100 grams of vegetables and 100 grams of fruits, while eliminating 50 grams of meat and 20 grams of cheese, would decrease NAE with about 15 mEq/d. Accordingly, serum bicarbonate levels would increase with about 0.5 mmol/l. This increase might seem marginal, however, it would imply a reduction of the prevalence of acidosis of 5%, or 40 RTR of our cohort achieving appropriate bicarbonate levels by simple dietary modification.

Conclusion

Acidosis is highly prevalent in RTR. In addition to conventional factors contributing to acidosis in RTR, diet appeared to be associated with acid-base homeostasis. Modification of the diet, by increasing fruit and vegetable intake and decreasing intake of animal protein, might improve acid-base balance in RTR.

Disclosures

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Urinary Sulfur Metabolites are Associated with a Favorable Cardiovascular Risk Profile and Survival Benefit in Renal Transplant Recipients

Submitted

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Abstract

Background The role of sulfur in post-transplant conditions is unclear. Sulfur may be protective by intermediate conversion to hydrogen sulfide (H_2S) and thiosulfate ($S_2O_3^{2-}$; TS). However, sulfate, the end-product of sulfur-containing amino-acids (SAA), contributes to metabolic acid load (NAE) and may adversely influence acid-base homeostasis. We investigated the association of urinary sulfur metabolites with cardio-metabolic parameters in renal transplant recipients (RTR), and analyzed their predictive capacity for mortality.

Methods Urinary sulfate and TS excretion was studied in 24h-urine from 707 RTR who were 5.4 [1.9-12.2] years after transplant surgery and 110 healthy controls. Diet was assessed for SAA content by food frequency questionnaires. Various cardio-metabolic risk factors, including hemodynamic parameters, Nt-pro-BNP, hs-CRP, HbA1c, eGFR, proteinuria and blood gases were measured.

Results Urinary sulfate was similar in RTR and controls while TS was higher in RTR than in controls. SAA intake was lower in RTR than in controls and significantly correlated with sulfate excretion but not with TS excretion. Sulfate was beneficially associated with eGFR, NAE, SBP, hs-CRP, Nt-Pro-BNP and proteinuria (all $p \leq 0.005$). TS was beneficially associated with eGFR, serum HCO_3^- and pH, hs-CRP and NT-Pro-BNP (all $p \leq 0.001$). During median follow-up of 27 [22-36] months, 47 RTR (7%) died. After adjustment for age, gender and renal function, Hazard Ratios for mortality were 0.87 (95%CI 0.82-0.92, $p<0.001$) and 0.60 (95% CI 0.41-0.59, $p=0.01$) for urinary sulfate and TS respectively.

Conclusion Urinary sulfate and TS excretion are beneficially associated with survival after renal transplantation, possibly by favorably influencing cardiovascular parameters. Despite a significant association of SAA with urinary sulfate and NAE, no adverse association was observed between sulfate and cardiovascular risk profiles and mortality. Intervention studies with exogenous sulfur are warranted to elucidate mechanisms underlying these beneficial and promising associations in RTR.

Introduction

Renal transplantation is the preferred treatment for patients with end stage renal disease. However, despite improvement of quality of life and life expectancy in renal transplant recipients (RTR), morbidity and mortality rates remain high compared with the general population [1, 2]. This likely has a multi-factorial origin since many risk factors, including cardiovascular disease, acidosis, low-grade inflammation and impaired graft function, are highly prevalent after transplantation [3-5]. A pressing need exists to gain insight in mechanisms underlying post-transplant morbidity and mortality to provide therapeutic tools for improvement of patient and graft survival. Previously, nutritional factors including sodium and metabolic acid load appeared to be adversely associated with various cardiovascular and metabolic processes in RTR [6, 7].

In the past decades, interest in sulfur metabolism in humans has grown considerably, particularly with regard to the synthesis and function of hydrogen sulfide (H_2S) [8-16]. H_2S is an endogenously produced gaseous compound and a signaling molecule with substantial biological potential, suggested to be beneficially involved in various (patho-) physiological processes including blood pressure regulation, inflammation, angiogenesis and cytoprotection during hypoxia [8-16]. H_2S is synthesized by conversion of the amino acid cysteine, which is either directly ingested via food or originating from the essential amino acid methionine. A specific intermediate of the H_2S metabolism is thiosulfate (TS, $S_2O_3^{2-}$), which is physiologically excreted in urine. Indeed, urinary TS has been reported to be linearly associated with inhaled or intravenously administered H_2S, and might therefore, at least in part, reflect systemic H_2S levels [17]. Because of the protective effects of H_2S, it can be hypothesized that increasing intake of sulfur containing amino acids (SAA) contributes to the synthesis of H_2S and beneficially influences the cardiovascular profile and, consequently, patient survival. On the other hand, the major end-product of SAA is sulfate (SO_4^{2-}) [18, 19] which is alleged adverse for its contribution to metabolic acid load and systemic acidosis, particularly in patients with impaired renal function [7]. Therefore, it remains unclear what the role of the sulfur metabolism is in cardiovascular and metabolic health in RTR and whether sulfuric compounds should be considered beneficial or harmful.

We investigated the association of sulfur containing metabolites with cardiovascular and metabolic parameters in a large, well-defined cohort of stable RTR. Intake of protein, particularly SAA, and protein sources was assessed with food frequency questionnaires. In RTR and in healthy controls, we measured sulfate and thiosulfate and investigated the associations of these metabolites with cardiovascular and metabolic parameters in RTR. Additionally, we analyzed the association of urinary sulfate and thiosulfate excretion with mortality in RTR.

Methods

Study populations

We invited all RTR (≥18 years) with a functioning graft for at least one year who visited our outpatient clinic between 2008 and 2010. RTR were all transplanted in the University Medical Center Groningen and had no history of drug or alcohol addiction. Of 817 initially invited RTR, 707 (87%) signed written informed consent to participate in this study. As a reference group, we included 110 subjects who were evaluated and approved for living kidney donation in our center. None had a history of kidney disease, diabetes or cardiovascular events. Hypertension, if present, was treated with a maximum of one antihypertensive drug. For analyses regarding dietary intake, we excluded patients with missing dietary data, resulting in 637 RTR eligible for analyses. The Institutional Review Board approved the study protocol (METc 2008/186), which was in adherence to the Declaration of Helsinki.

Assessment of dietary intake

Dietary intake was assessed with a validated semi-quantitative food frequency questionnaire (FFQ) that was developed at Wageningen University [20] and updated several times. For our study, the FFQ was slightly modified for accurate assessment of protein intake, including types and sources of protein. Validity of the FFQ in RTR was assessed as described previously [21]. The FFQ inquired about intake of 177 food items during the last month taking seasonal variations into account. For each item, the frequency was recorded in times per day, week, or month. The number of servings was expressed in natural units (e.g. slice of bread or apple) or household measures (e.g. cup or spoon). The questionnaire was self-administered and filled out at home. All FFQs were checked for completeness by a trained researcher and inconsistent answers were verified with the patients. Dietary data were converted into daily nutrient intake using the Dutch Food Composition Table of 2006 [22].

Urine and plasma parameters

All participants were instructed to collect a 24-hour urine sample according to a strict protocol at the day prior to their visit to the outpatient clinic. Urine was collected under oil and chlorhexidin was added as antiseptic agent. Urinary sulfate was measured chromatographically (Metrohm, type 861, Herisau, Switzerland). Urinary thiosulfate was determined by a specific HPLC method as described previously [23, 24]. Briefly, 25µL of urine was derivatized with 5 µl of 46 mM monobromobimane, 25 µl of acetonitrile, and 25 µl of 160 mM HEPES/16 mM EDTA pH 8 buffer (Invitrogen, Carlsbad, CA) for 30 minutes in the dark. Derivatization of thiol groups was stopped by 50 µl of 65

mM methanosulfonic acid (Fluka, Buchs, Switzerland) and proteins were removed by recentrifugation. Electrolytes (chloride, potassium, sodium, calcium and phosphate) were directly analyzed using standard laboratory procedures. Net acid excretion (NAE), the gold standard for assessing metabolic acid load, was calculated as titratable acid (TA) + ammonium (NH_4^+) – bicarbonate (HCO_3^-) [7,25]. TA was measured with an automated titrator (Metrohm, 855 Robotic Titrosampler, Switzerland), NH_4^+ and HCO_3^- were measured chromatographically (Waters, Alliance HT 2795 and Metrohm, type 861, Herisau, Switzerland respectively). Blood was drawn in the morning after completion of the 24h urine collection. Venous blood gas analyses were assessed photometrically immediately after collection of blood samples. Plasma and urinary concentrations of electrolytes, phosphate, albumin and urea were measured using routine laboratory methods, as were serum cholesterol, HbA1c, hs-CRP and Nt-Pro-BNP-levels. Serum creatinine was determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Germany). Renal function was assessed by estimating glomerular filtration rate (eGFR) applying the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [26]. Urinary albumin concentration was determined by nephelometry (Dade Behring Diagnostic, Marburg, Germany). Total urinary protein concentration was analyzed using the Biuret reaction (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Proteinuria was defined as urinary protein excretion ≥0.5 g/24h.

Clinical parameters

All measurements were performed during a morning visit to the outpatient clinic after an 8-12h overnight fasting period. Blood pressure (mmHg) was measured according to a strict protocol as previously described [6]. Participants were left alone in a room in half-sitting position while systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate were measured with a semi-automatic device (Dinamap® 1846, Critikon, Tampa, FL, USA). Measurements were performed every minute for fifteen minutes and the last three values were averaged. Information on participants' health status, medical history, and medication use was obtained from patient records. Information on smoking behavior was obtained by using a questionnaire. Participants were classified as current, former or never smokers. Body weight and height were measured with participants wearing indoor clothing without shoes. BMI was calculated as weight divided by height squared (kg/m^2) and Body Surface Area (BSA) was estimated applying the universally adopted formula of DuBois and DuBois [27].

Statistical analyses

Data-analysis was performed using SPSS 18.0 (Chicago, IL, USA). Normality was tested with the Kolmogorov-Smirnov test and skewed data were normalized for analyses by

logarithmic transformation (albuminuria, thiosulfate excretion, hs-CRP, Nt-Pro-BNP, protein excretion). Data are presented as mean ± standard deviation (SD), unless stated otherwise. Differences between RTR and healthy controls were tested with the t-test for independent samples, the Mann-Whitney-*U*-test or the chi-square test. The associations of (thio)sulfate-excretion with various cardiovascular factors were analyzed with linear regression analysis, with adjustment for age, gender and BSA (model 1) and cumulative additional adjustments for eGFR (model 2) and urinary sodium excretion and use of medication (antihypertensives, calcineurin inhibitors, proliferation inhibitors; model 3). To study the potential interdependence of both sulfur metabolites, we also adjusted for either thiosulfate or sulfate (model 4). The association of dietary intake with urinary sulfur metabolites was investigated with linear regression analyses adjusting for age and gender (model 1), BSA (model 2) and eGFR (model 3). Regression coefficients are given as beta or standardized beta, the latter referring to the number of SD a dependent variable changes, per SD increase of the independent variable (either sulfate or thiosulfate), allowing for comparison of the strength of the associations. In prospective analyses, we investigated associations of urinary sulfate and thiosulfate excretion with mortality in RTR. We performed crude Cox regression analyses (model 1) and analyses with adjustment for age, gender (model 2) and renal function (model 3). In all analyses, a two-sided P value less than 0.05 was considered statistically significant.

Results

The characteristics of RTR and controls are shown in *table 1*. For RTR, median time between transplantation and baseline measurements was 5.4 [1.9-12.2] years. The two groups were similar with respect to age, BMI and BSA. Men were overrepresented in the RTR-group compared to the control group. Urinary sulfate excretion did not differ between both groups (18.0±5.6 in controls vs 17.6±6.4 in RTR; p=0.29), but thiosulfate excretion was significantly higher in RTR compared to controls (7.0 [3.9-11.9] vs 2.1 [0.29-10.1]; p<0.001). As anticipated, creatinine clearance was significantly lower in RTR than in healthy subjects (66±26 vs. 132±41 ml/min; p<0.001). Correspondingly, higher blood pressure values were observed in RTR than in controls (SBP 136±18 vs. 125±15 mmHg, DBP 83±11 vs. 75±9 mmHg; both p<0.001), despite 89% of RTR using one or more antihypertensive drugs. Compared to controls, RTR had significantly higher serum levels of triglycerides, glycosylated hemoglobin (HbA1c), hs-CRP and Nt-pro-BNP (all p<0.001). Serum albumin levels, serum pH and serum bicarbonate levels were lower and RTR were more likely to have metabolic acidosis (blood HCO_3^-< 24 mmol/l; p<0.001). Energy intake, as well as intake of total protein, plant protein, SAA and total fat was significantly lower in RTR than in controls.

Table 1 Characteristics of 110 healthy controls and 707 renal transplant recipients at the day of their visit to the outpatient clinic.

Characteristics	Healthy controls (n =110)	RTR (n = 707)	p diff.
Urinary sulfate (mmol/24h)	18.0 ± 5.6	17.6 ± 6.4	0.29
Urinary thiosulfate (umol/24h)	2.1 [0.29-10.1]	7.0 [3.9-11.9]	<0.001
Demographics			
Age (years)	53± 10	53 ± 13	0.68
Male gender, n (%)	47	57	0.003
BMI (kg/m^2)	26.5 ± 3.4	26.7 ± 4.8	0.73
BSA (m^2)	1.97 ± 0.19	1.94 ± 0.22	0.20
Smoking behavior (current, %)	24	13	<0.001
Medication use			
Anti-hypertensives, %	17	88	<0.001
Statins, %	6	53	<0.001
Hemodynamic parameters			
SBP, mmHg	125 ± 15	136 ± 18	<0.001
DBP, mmHg	75 ± 9	83 ± 11	<0.001
MAP, mmHg	92 ± 10	100 ± 12	<0.001
Heart Rate, bpm	67 ± 10	69 ± 12	0.07
Renal Function parameters			
Serum Creatinine, umol/L	73 [64-82]	125 [100-160]	<0.001
eGFR, ml/min	93 ± 13	52 ± 20	<0.001
Proteinuria (≥ 0.5 g/d), %	0.5	24	<0.001
Albumin excretion	5.5 [3.1-8.3]	41.6 [10.6-179]	<0.001
Serum parameters			
Albumin, mmol/L	45.2 ± 2.5	43.0 ± 3.0	<0.001
Cholesterol, mmol/L	5.3 [4.5-5.9]	5.0 [4.3-5.8]	0.14
Triglycerides, mmol/L	1.1 [0.8-1.6]	1.7 [1.3-2.3]	<0.001
HbA1c, %	5.55 ± 0.28	5.99 ± 0.83	<0.001
hsCRP,mg/L	1.2 [0.5-2.2]	1.6 [0.7-4.6]	0.001
Nt-pro-BNP, ng/L	38 [20-65]	254 [109-614]	<0.001
Venous blood gas analyses			
pH, kPa	7.38 ± 0.04	7.37 ± 0.04	0.02
pCO2, kPa	6.07 ± 0.79	5.85 ± 0.80	0.02
HCO3-, mmol/L	25.9 ± 1.7	24.6 ± 3.1	<0.001
Acidosis, %*	8	31	<0.001
Dietary Intake (FFQ)*			
Energy (kCal/d)	2335 ± 687	2175 ± 638	0.02
Protein, g/d	88 ± 23	82 ± 20	0.01
Animal protein, g/d	54 ± 17	52 ± 15	0.12
Plant protein, g/d	34 ± 10	30 ± 10	0.003
S-AA, g/d	2.63 ± 0.80	2.41 ± 0.68	0.01
Total Fat, g/d	96 ± 36	88 ± 34	0.05
Total Carbohydrates, g/d	263 ± 79	249 ± 78	0.08

Data are presented as mean ± SD, % or median [interquartile range]. Abbreviations: RTR: renal transplant recipients; BMI, body mass index; BSA, body surface area, SBP systolic blood pressure; DBP diastolic blood pressure; MAP, mean arterial pressure; eGFR, estimated Glomerular Filtration Rate; HbA1c glycosylated hemoglobine; hsCRP high-sensitive C-Reactive Protein; Nt-pro-BNP N-terminal pro-Brain Natriuretic Peptide; FFQ food frequency questionnaire; S-AA sulfur containing amino acids. * Acidosis was defined as blood HCO_3^- < 24 mmol/L. P for difference was tested by the independent t-test, kruskal-wallis test or χ^2-test. *data available in 637 of 707 RTR and in 110 controls.

Table 2a Regression coefficients for the association of urinary sulfate and thiosulfate with cardiovascular parameters in 707 renal transplant recipients.

Dependent variable	Model 1		Model 2		Model 3		Model 4	
	β	p	β	p	β	p	β	p
Sulfate								
SBP, mmHg	-0.21	0.06	-0.21	0.05	-0.34	0.004	-0.33	0.008
DBP, mmHg	0.04	0.52	0.05	0.51	-0.01	0.90	-0.01	0.98
MAP, mmHg	-0.04	0.62	-0.04	0.61	-0.12	0.15	-0.11	0.20
Pulse pressure, mmHg	-0.25	0.002	-0.26	0.004	-0.33	<0.001	-0.32	<0.001
Heart rate, bpm	-0.13	0.09	-0.16	0.04	-0.15	0.08	-0.14	0.11
Nt-pro-BNP*, ng/L	-0.05	<0.001	-0.04	<0.001	-0.03	<0.001	-0.03	0.001
hsCRP*, mg/L	-0.03	<0.001	-0.03	<0.001	-0.04	<0.001	-0.03	0.002
HbA1c, %	-0.01	0.13	-0.02	0.03	-0.02	0.007	-0.01	0.02
eGFR, ml/min	0.59	<0.001	*	*	0.48	<0.001	0.31	0.006
Proteinuria*, g/24h	-0.05	0.02	-0.03	0.19	-0.04	0.002	-0.04	<0.001
Thiosulfate								
SBP, mmHg	-0.05	0.16	-0.03	0.27	-0.03	0.22	0.02	0.69
DBP, mmHg	0.01	0.92	0.01	0.85	0.01	0.87	0.01	0.82
MAP, mmHg	-0.01	0.34	-0.01	0.93	-0.01	0.92	0.02	0.77
Pulse pressure, mmHg	-0.05	0.37	-0.04	0.46	-0.04	0.47	0.01	0.82
Heart rate, bpm	-0.06	0.69	-0.10	0.12	-0.09	0.14	-0.07	0.26
Nt-pro-BNP*, ng/L	-0.03	<0.001	-0.03	<0.001	-0.03	<0.001	-0.02	0.01
hsCRP*, mg/L	-0.02	<0.001	-0.01	<0.001	-0.02	<0.001	-0.01	0.01
HbA1c, %	-0.01	0.48	-0.01	0.18	-0.01	0.19	-0.01	0.55
eGFR, ml/min	0.43	<0.001	*	*	0.34	<0.001	0.28	<0.001
Proteinuria*, g/24h	-0.02	0.001	-0.01	0.08	-0.01	0.06	-0.01	0.31

Table 2b Regression coefficients for the association of urinary sulfate and thiosulfate with metabolic parameters in 707 renal transplant recipients.

Dependent variable	Model 1		Model 2		Model 3		Model 4	
	β	p	β	p	β	p	β	p
Sulfate								
NAE, mEq/24h	1.49	<0.001	1.37	<0.001	1.33	<0.001	1.42	<0.001
Venous HCO_3^- mmol/L	0.07	0.002	0.04	0.11	0.03	0.13	0.01	0.53
Venous pH	0.001	0.01	0.001	0.33	0.001	0.55	0.001	0.70
Thiosulfate								
NAE, mEq/24h	0.21	0.02	0.11	0.21	0.09	0.31	-0.11	0.02
Venous HCO_3^- mmol/L	0.05	<0.001	0.03	<0.001	0.03	<0.001	0.02	0.004
Venous pH	0.01	<0.001	0.01	<0.001	0.01	<0.001	0.01	0.001

Coefficients are provided as standardized betas, referring to the number of standard deviations the dependent variable changes, per standard deviation increase of (thio)sulfate. Model 1: adjusted for age, gender and BSA, model 2: additionally adjusted for eGFR and time since transplantation, model 3: additionally adjusted for UNaV, drugs (antihypertensives, CNI, proliferation inhibitors), model 4: additionally adjusted for either sulfate or thiosulfate. *Log-transformed for analyses.Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; hs-CRP, high-sensitive CRP; NAE, net acid excretion; eGFR, estimated glomerular filtration rate.

The regression coefficients for the associations of urinary sulfate and thiosulfate with cardiovascular parameters are presented in *table 2a.* After adjustment for potential confounders (model 3), urinary sulfate excretion was significantly inversely associated with SBP (β=-0.34; p=0.004), pulse pressure (β=-0.33; p<0.001), serum Nt-pro-BNP (β=-0.03; p<0.001), serum hs-CRP (β=-0.04; p<0.001), serum HbA1c (β=-0.02; p=0.007) and proteinuria (β=-0.04; p=0.002). A direct association was observed between urinary sulfate excretion and eGFR (β=0.48; P<0.001). All observed associations remained significant after additional adjustment for urinary thiosulfate excretion. Thiosulfate excretion was significantly inversely associated with Nt-pro-BNP (β=-0.03; p<0.001), and hs-CRP (β=-0.02; p<0.001), also after adjustment for sulfate excretion (model 4). A direct association was observed between thiosulfate and eGFR (β=0.34; p<0.001. Associations of sulfate and thiosulfate with metabolic parameters are shown in *table 2b*. Sulfate was positively associated with NAE (β=1.33; p<0.001), reflecting metabolic acid load, but no association was seen with serum HCO_3^- β=1.03; p=0.13) or serum pH (β=0.001; p=0.55). For thiosulfate, no association was observed with NAE, however direct associations were observed with venous HCO_3^- (β=0.03; p<0.001) and venous pH (β=0.01; p<0.001). Again, these associations remained significant after additional adjustment for urinary sulfate excretion. The standardized betas of the associations of sulfur containing metabolites with cardiovascular and metabolic parameters (model 3), allowing for mutual comparison of the strengths of associations, are depicted in *Figure 1a* and *Figure 1b* respectively.

The regression coefficients for the association of dietary (protein) and urinary (thio) sulfate excretion are shown in *table 3*. After adjustment for age, gender, BSA and renal function, significant positive associations were observed between urinary sulfate and intake of total protein (St. β=0.22; p<0.001), animal protein (St. β=0.23; p<0.001), vegetable protein (St. β=0.08; p=0.03) and intake of SAA (St. β=0.20-0.24; p<0.001). No such associations were observed between dietary protein and urinary thiosulfate excretion.

Figure 1a Regression coefficients for the association of urinary sulfate and thiosulfate excretion with cardiovascular parameters. Associations are adjusted for age, gender, BSA, eGFR, time since transplantation, UNaV and drugs (antihypertensives, CNI, proliferation inhibitors). Regression coefficients are given as standardized betas, referring to the number of SD the cardiovascular parameter changes per SD increase of either sulfate or thiosulfate.

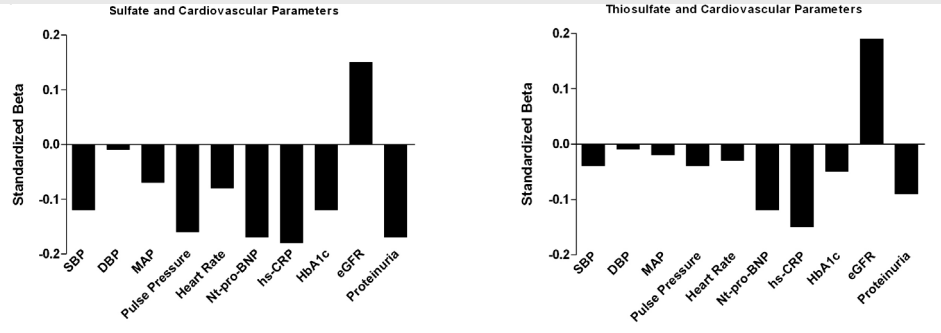

Figure 1b Regression coefficients for the association of urinary sulfate and thiosulfate excretion with metabolic parameters. Associations are adjusted for age, gender, BSA, eGFR, time since transplantation, UNaV and drugs (antihypertensives, CNI, proliferation inhibitors). Regression coefficients are given as standardized betas, referring to the number of SD the metabolic parameter changes per SD increase of either sulfate or thiosulfate.

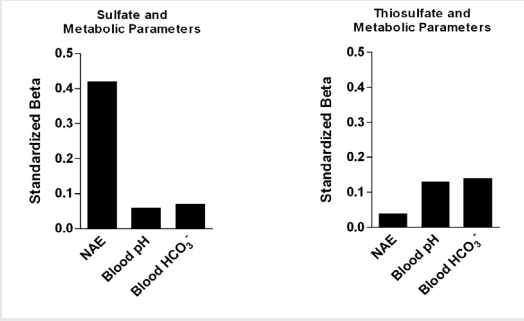

Table 3 Regression coefficients for the association of protein intake with urinary sulfate and thiosulfate excretion in 637 RTR

	Model 1		Model 2		Model 3	
	St. β	p	St. β	p	St. β	p
Sulfate						
Total protein (g/d)	0.25	<0.001	0.23	<0.001	0.22	<0.001
Animal protein (g/d)	0.27	<0.001	0.24	<0.001	0.23	<0.001
Plant protein (g/d)	0.10	0.011	0.09	0.018	0.08	0.032
Methionine (mg/d)	0.27	<0.001	0.24	<0.001	0.24	<0.001
Cysteine (mg/d)	0.23	<0.001	0.21	<0.001	0.20	<0.001
Methionine + Cysteine (mg/d)	0.25	<0.001	0.23	<0.001	0.22	<0.001
Thiosulfate						
Total protein (g/d)	0.01	0.85	0.02	0.72	0.01	0.85
Animal protein (g/d)	-0.02	0.66	-0.01	0.80	-0.01	0.74
Plant protein (g/d)	0.05	0.28	0.05	0.26	0.04	0.37
Methionine (mg/d)	-0.02	0.67	-0.01	0.79	-0.02	0.68
Cysteine (mg/d)	0.01	0.81	0.02	0.72	0.01	0.88
Methionine + Cysteine (mg/d)	0.01	0.92	0.01	0.81	0.002	0.96

Coefficients are provided as standardized betas, referring to the number of standard deviations (thio)sulfate changes, per standard deviation increase of the nutrient.
Model 1: adjusted for age and gender; model 2: additionally adjusted for BSA; model 3: additionally adjusted for eGFR (CKD-EPI

Sulfate, thiosulfate and mortality

Median follow-up from baseline was 27 [22-36] months. During this prospective follow-up, 47 (7%) RTR died. RTR who died had significantly lower urinary excretion of sulfate and thiosulfate than RTR who survived during follow-up (13.2±5.5 vs 17.9±6.3 mmol/24h, p<0.001 for sulfate and 5.4 [2.8-8.5] vs 7.2 [4.0-12.1] umol/24h, p=0.01 for thiosulfate). According to gender-stratified tertiles of sulfate, incidence of mortality during follow-up was 31 out of 203 (15%) for the lowest tertile, while this was 10 out of 226 (4%) and 6 out of 229 (3%) in the middle and highest tertiles respectively (log-rank test p<0.001, figure 2a). According to increasing gender-stratified tertiles of thiosulfate, these numbers were 21 (10%), 16 (7%) and 7 (3%) respectively (log-rank test p=0.02, figure 2b). Results of Cox regression analyses for mortality in RTR are shown in table 4. After adjustment for age, gender and renal function (model 3), sulfate and thiosulfate were significantly associated with mortality with hazard ratios of 0.87 [0.82-0.92], p<0.001 and 0.60 [0.41-0.89], p=0.01 respectively.

Figure 2 Kaplan-Meier curves for patient survival according to urinary sulfate (a) and thiosulfate (b) excretions.

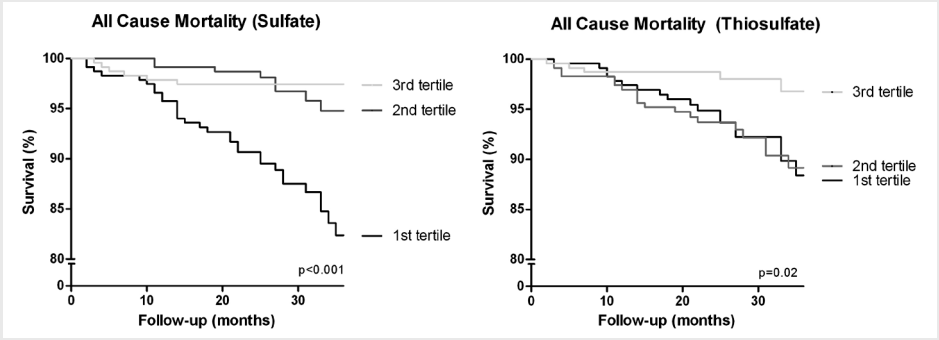

Table 4 Cox regression analyses for prediction of patient mortality based on urinary excretion of sulfate and thiosulfate

	Sulfate			Thiosulfate		
	HR	**[95% CI]**	**p**	**HR**	**[95% CI]**	**p**
Model 1	0.87	[0.83-0.92]	<0.001	0.59	[0.42-0.85]	0.004
Model 2	0.86	[0.81-0.91]	<0.001	0.55	[0.38-0.80]	0.002
Model 3	0.87	[0.82-0.92]	<0.001	0.60	[0.41-0.89]	0.01

Thiosulfate was log-transformed for analyses.
Model 1: crude model;
Model 2 adjusted for age and gender;
Model 3, as model 2, additionally adjusted for renal function (CKD-EPI)

Discussion

The cardinal finding of the present study is the significant association of urinary sulfur metabolites with a favorable cardiovascular risk profile and with improved survival in RTR. These associations were found despite the observed direct association of urinary sulfate with the allegedly adverse metabolic acid load. Also, RTR had a markedly elevated excretion of urinary thiosulfate, but not sulfate, compared with healthy controls. Our findings suggest a protective role for sulfur metabolites in cardiovascular changes following renal transplantation and in patient survival long-term after transplantation.

Humans depend on exogenous sources for their sulfur supplies as sulfur enters the body mainly as a constituent of the sulfur containing amino acids (SAA) cysteine and methionine. In line with previous studies, we observed a significant direct association between intake of SAA and urinary sulfate excretion [18, 19]. SAA are eventually converted to sulfate and are allegedly adverse for their contribution to metabolic acid load, which has previously been shown to be associated with the extent of acidosis in RTR [7]. In accordance, a significant association between intake of SAA and net acid excretion was found, the latter reflecting metabolic acid load [25]. Nevertheless, regarding systemic metabolic parameters, we observed no significant association of sulfate with blood pH and HCO_3^-. The opposite held true for TS, showing significant direct associations with both blood pH and HCO_3^-, which could suggest a favorable role for TS in acid-base homeostasis, given the large body of RTR experiencing systemic acidosis[7]. The significance and mechanism underlying this association is not yet clear and needs further elucidation.

Regarding cardiovascular parameters, we observed beneficial associations of TS with Nt-Pro-BNP, hs-CRP and eGFR. Since TS is a central metabolite in the metabolic pathway of H_2S, a gaseous transmitter known for its protective role in several pathological processes such as inflammation and hypertension, a potential explanation for our findings might be involvement of H_2S. H_2S is synthesized endogenously by the activity of three major enzymes, cystathionine-β-synthetase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MPST) that all use the amino acid L-cysteine as their main substrate [28]. CSE, the most predominant enzyme in cardiovascular tissue, is expressed in endothelial and smooth muscle cells. In the vasculature, H_2S is known to function as an endothelial cell-relaxing factor. Accordingly, $CSE^{-/-}$ mice have a slightly higher blood pressure than wild-type mice [29]. Endothelial derived H_2S also functions as a promoter of angiogenesis and as an inhibitor of leukocyte adhesion, thereby reducing inflammation [8, 14]. Recently, Tokuda *et al* reported that inhalation of H_2S prevented endotoxin-induced systemic inflammation and even prolonged survival in endotoxaemic mice [30]. H_2S eventually gets degraded to TS, which is partly re-used as sulfur donor to form a new H_2S molecule, partly converted to sulfite, the latter being oxidized to sulfate and physiologically excreted in the urine. A certain amount of TS however, is excreted directly in the urine, possibly as 'spillover' of H_2S synthesis and might therefore at least in part reflect systemic levels of H_2S [31]. Another potential explanation for our findings might be that TS itself underlies the favorable associations with cardiovascular parameters in RTR. TS has been reported to exert protective activities in several pathophysiological processes such as oxidative stress [32]. Also, TS has recently been shown to completely prevent vascular calcifications in uremic rats [33]. Moreover, TS can safely be administered to humans and is well tolerated [23].

Unexpectedly, similar beneficial associations were observed for sulfate, with even additional favorable associations with SBP, pulse pressure, HbA1c and proteinuria. This was in contrast with our hypothesis that urinary sulfate, reflecting intake of acidifying SAA and metabolic acid load, contributes to systemic acidosis and related complications like hypertension and insulin resistance, particularly in patients with impaired renal function[7]. An explanation might be that part of the excreted sulfate has been incorporated in the H_2S and/or TS synthesis as well, which is in line with previous studies showing that systemically applied H_2S is excreted as either TS or sulfate [31, 34]. This could also explain our findings of survival benefit in RTR with either higher urinary sulfate or higher TS excretion.

In our study, unlike sulfate, thiosulfate was not associated with dietary sulfur intake. Although the synthesis of H_2S, and consequently TS, depends on the intake of SAA, particularly methionine, the rate-limiting step is the presence of specific enzymes such as CBS and CSE. The absence of any association of TS with dietary intake could be explained by an excess presence of SAA. We observed significantly higher urinary excretion of thiosulfate in RTR compared with healthy controls. This was quite surprising, since it might be expected that RTR, known to be at high cardiovascular risk compared to the general population, excrete less of an indicator of the allegedly favorable H_2S metabolism. It seems unlikely that differences in dietary (protein) intake between both groups underlie the differences in urinary TS excretion, since intake was lower in RTR compared with controls. Possibly, the higher urinary thiosulfate excretion reflects a compensatory response to meet the increased demand for cardiovascular protection in RTR. It could also be that prednisolone treatment in RTR explains the difference, since it is known that glucocorticoids induce CBS activity in the liver [35].

Strengths of our study include the large sample size of this specific patient group consisting of well defined, stable RTR. Extensive data collection, including data from 24h urine samples and dietary intake allowed for adjustment for many confounders. We acknowledge, however, that there are also limitations. The present study is an observational epidemiological study, which makes it difficult to conclude on causality. Since little is known on the role of sulfur in cardiovascular health in RTR and the sulfur handling in the kidney, a third factor might underlie the observed associations. For example, it can be hypothesized that higher urinary excretion of sulfur compounds is caused by decreased tubular reabsorption due to renal damage, which itself is well known to be associated with cardiovascular risk. However, the association between urinary sulfur metabolites and cardiovascular parameters remained significant in multivariate regression analyses, including renal function, suggesting that this association is independent of confounding factors. Nevertheless, residual confounding could have remained, since it is hard to completely adjust for the severity of each risk factor. Additionally, our study population consisted predominantly of Caucasian people, which calls prudence to extrapolation of our results to populations of other ethnicities. The detailed metabolic determinants influencing urinary excretions of both sulfate and TS are not clear thus far and require further elucidation. Additional studies could focus on the H_2S and TS metabolism and the involved enzymes, to reveal the mechanism underlying the observed associations and to investigate the potentially beneficial effects of sulfur suppletion.

Conclusion

Post-transplant conditions are associated with markedly increased risk for cardiovascular diseases compared with healthy individuals. The observed beneficial associations of urinary sulfur metabolites with cardiovascular parameters and patient survival in our transplant cohort might point towards a protective role of components in the sulfur metabolism. Long-term intervention studies with exogenous sulfur, either from the diet or pharmacological agents such as sodium thiosulfate, are warranted to clarify the exact role and underlying mechanisms of the sulfur metabolism in cardiovascular health and survival in RTR.

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6

Reduced Urinary NO_2/NO_3-excretion is Associated with an Adverse Cardiovascular Risk Profile in Renal Transplant Recipients

In preparation

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Abstract

Background Cardiovascular risk is high in renal transplant recipients (RTR) and a leading cause for graft loss and mortality. Since endothelial dysfunction is common in RTR, reduced nitric oxide production might relate to an increased cardiovascular risk profile in RTR. Aims of this study were to investigate nitric oxide production in RTR vs controls, to identify determinants of nitric oxide production in RTR and the association of nitric oxide with cardiovascular risk factors in RTR.

Methods We included 707 outpatient RTR (age 53±13 yrs, 57% male, time since transplantation 5.4 [1.9-12.2] yrs) and 107 healthy controls (53±10 yrs, 47% male). Systemic NO production was assessed by measurement of 24h urinary excretion of its stable end products NO_2/NO_3 (NO_x). Detailed data on diet (questionnaires) and drugs were obtained to assess exogenous NO-sources (mainly vegetables). Cardiovascular risk factors (blood pressure, pulse pressure, heart rate, Nt-pro-BNP, hs-CRP, HbA1c, renal function, albuminuria) were measured.

Results NO_x was markedly lower in RTR than in controls (588 [400-812] vs. 1020 [796-1285] nmol/24h, p<0.001), while vegetable intake was similar (92±57 vs. 93±58 g/d, p=0.9) albeit approximately half of the WHO-recommended intake. Gender, vegetable intake and renal function were independent determinants of NO_x in RTR. Systolic and diastolic BP were 136±18 and 83±11 mmHg respectively; creatinine clearance was 66±26 ml/min. In linear regression analyses, urinary NO_x excretion was inversely associated with SBP (β=-0.29; p=0.005), pulse pressure (β=-0.16; p=0.02), heart rate (β=-0.19 p=0.008), hs-CRP (β=-0.02 p=0.008), Nt-pro-BNP (β=-0.003, p<0.001) and HbA1c (β=-0.01 p=0.05) and positively with creatinine clearance (β=0.51, p=0.002), independent of age, sex, body surface area, drug use and vegetable intake. No association was observed with albuminuria.

Conclusion The pronounced reduction in urinary NO_x excretion in RTR compared to controls, and its robust, independent association with an adverse cardiovascular risk profile points towards a role for endogenous nitric oxide in increased cardiovascular risk in RTR.

Introduction

Worldwide, the prevalence of end stage renal disease (ESRD) is increasing rapidly, without indication of a slowing down in rate[1]. Kidney transplantation is the preferred treatment for patients with ESRD since it reduces mortality risk and improves quality of life for patients on dialysis[2]. However, even after successful transplantation, both long term allograft and patient survival are limited. The main reason for these poor outcomes is the high prevalence of post-transplant cardiovascular disease, eventually leading to graft loss or death[3, 4]. Another reason is the gradual decline in graft function due to chronic renal transplant dysfunction, ultimately requiring re-transplantation or return to dialysis[5, 6].

The gaseous transmitter nitric oxide (NO) is considered a crucial signaling molecule involved in various physiological processes like regulation of vascular tone[7], adhesion of inflammatory cells[8], smooth muscle cell proliferation[9] and platelet aggregation[10]. Endothelial NO synthase (eNOS) is the major enzyme in the vascular wall producing NO and inhibition of this enzyme in experimental animal studies readily results in glomerular and systemic hypertension[11]. Also, findings from experimental studies in renal tissue of patients following kidney transplantation suggest a role for NO in acute and chronic transplant rejection[12, 13]. In several other patho-physiological human conditions such as hypercholesterolemia, atherosclerosis and hypertension both impaired eNOS and endothelial dysfunction are observed, suggesting a causative link between those two factors[14-16]. Whether NO also plays a role in the increased cardiovascular risk and endothelial dysfunction long-term after renal transplantation remains to be elucidated.

Some recent studies suggest that NO can be converted from exogenous inorganic nitrate, mainly originating from vegetables, and that nitrate supplementation may have therapeutic effects in renal and cardiovascular disease[17, 18]. Since prior to renal transplantation dialysis patients receive stringent dietary guidelines to restrict potassium intake by reducing intake of vegetables, it can be hypothesized that potential NO deficiency in RTR occurs due to perseverance with these dietary guidelines after transplantation.

In this study, we compared urinary NOx-excretion, reflecting NO-production, between RTR and healthy controls. Second, we studied whether potential differences in urinary NOx-excretion could be explained by differences in dietary habits. Third, we investigated whether urinary NOx-excretion was associated with the cardiovascular risk profile in RTR.

Methods

Study populations

We invited all RTR (≥ 18 years) with a functioning graft for at least one year who visited our outpatient clinic between 2008 and 2010. RTR were all transplanted in our center and had no history of drug or alcohol addiction. Of 817 initially invited RTR, 707 (87%) signed written informed consent to participate in this study. As a healthy reference group, we included 107 subjects who were evaluated for living kidney donation in our center during the same period. None had a history of kidney disease, diabetes or cardiovascular events. Hypertension, if present, was treated with a maximum of one antihypertensive drug. For analyses regarding dietary intake, we excluded all patients with missing dietary data, resulting in 637 RTR and 95 healthy controls for the analyses. The Institutional Review Board approved the study protocol (METc 2008/186), which was in adherence to the Declaration of Helsinki.

Assessment of nitric oxide production

NO has a short biological half-life and is rapidly converted into its stable metabolites, nitrite (NO_2) and nitrate (NO_3), that both are physiologically excreted in the urine. Measurement of combined urinary nitrite and nitrate (NOx) excretion is widely used as a marker of NO production [19, 20]. We colorimetrically measured urinary NOx excretion applying the Griess reaction after reduction of nitrate to nitrite in 24-hour urine samples. All participants collected urine according to the gold standard. In advance of the urine collection, an antiseptic agent (chlorhexidine) was added to the urine container to prevent bacterial growth and subsequent increased amounts of nitrite [21].

Assessment of dietary intake

Dietary intake was assessed using a validated semi quantitative food frequency questionnaire (FFQ) that inquired about intake of 177 food items during the last month, taking seasonal variations into account. For each item, the frequency was recorded in times per day, week, or month. The number of servings was expressed in natural units (e.g. slice of bread or apple) or household measures (e.g. cup or spoon). The questionnaire was self-administered and filled out at home. All FFQs were checked for completeness by a trained researcher and inconsistent answers were verified with the patients. Validity of the FFQ in RTR was assessed as described previously [22]. Dietary data were converted into daily nutrient intake using the Dutch Food Composition Table of 2006 [23].

Outcome measures

All measurements were performed during a morning visit to the out-patient clinic after an 8-12h overnight fasting period. Blood pressure (mmHg) was measured according to a strict protocol as previously described[24]. Participants were left alone in a room in half-sitting position while systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate were measured with a semi-automatic device (Dinamap® 1846, Critikon, Tampa, FL, USA). Measurements were performed every minute for fifteen minutes and the average value of the last three measurements was used in the analyses. Blood was drawn in the morning after completion of the 24h urine collection. Plasma and urinary concentrations of electrolytes, phosphate, albumin and urea were measured using routine clinical laboratory methods, as were serum cholesterol, HbA1c, hs-CRP and Nt-Pro-BNP-levels. Serum creatinine was determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Renal function was assessed by calculating the estimated glomerular filtration rate (eGFR) applying the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [25]. In a subgroup consisting of 201 RTR, GFR was measured by constant low dose infusion of the radio-labeled tracer ^{125}I-iothalamate, as described by Visser and Apperloo et al. [26, 27]. Simultaneously, effective renal plasma flow (ERPF) was measured as the clearance of ^{131}I-hippurate. Filtration fraction (FF) was calculated as GFR/ERPF x 100.

Information on participants' health status, medical history, and medication use was obtained from patient records. Information on smoking behavior was obtained by using a questionnaire. Participants were classified as current smokers, former smokers, or never smokers. Body weight and height were measured with participants wearing indoor clothing without shoes. Body Mass Index (BMI) was calculated as weight divided by height squared (kg/m^2) and Body Surface Area (BSA) was estimated applying the universally adopted formula of DuBois and DuBois [28].

Statistical analyses

Data-analysis was performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). Normality was tested with the Kolmogorov-Smirnov test and skewed data were normalized for analyses by either logarithmic transformation (urinary albumin excretion, hs-CRP and Nt-pro-BNP) or by extracting the square root of original values (24h urinary NOx-excretion). Data in text and tables are presented as mean ± standard deviation (SD), unless stated otherwise. Differences between RTR and healthy controls were tested with the t-test for independent samples, the Mann-Whitney-*U*-test or the chi-square test. To visualize potential associations of NOx-excretion with several parameters in RTR, we divided the study population into gender-stratified tertiles of NOx-excretion. P-trends

over tertiles of NOx-excretion were then obtained using univariate regression analyses, Kruskal-Wallis test or chi-square test. The associations of several cardiovascular factors with NOx-excretion were investigated with multivariable linear regression analysis, with adjustment for age (continuous) and gender (Model 1), subsequently for BSA (continuous; Model 2) and eGFR (continuous; Model 3). In model 4, an additional adjustment was made for urinary magnesium reflecting intake of vegetables. Linear regression coefficients are given as standardized betas (st. β), to allow for comparison of the regression coefficients. Within all statistical analyses, a two-sided P value less than 0.05 was considered statistically significant.

Results

The characteristics of RTR and healthy controls are shown in *table 1.* The two groups were similar with respect to age, BMI and BSA. Men were overrepresented in the RTR-group compared to the healthy controls. Urinary NOx-excretion was significantly lower in the RTR group than in the healthy controls (median [interquartile range] 588 [400-812] nmol/24h versus 1020 [796-1285] nmol/24h, $p<0.001$). As anticipated, creatinine clearance was significantly lower in RTR than in healthy subjects (66±26 vs. 132±41 ml/min; $p<0.0001$). Accordingly, blood pressure was significantly higher in RTR than in controls (SBP 136±18 vs. 125±15 mmHg, $p<0.001$; DBP 83±11 vs. 75±9; $p<0.001$), despite 89% of RTR using one or more antihypertensive drugs. Compared to healthy controls, RTR had significantly higher serum levels of triglycerides, glycated hemoglobin (HbA1c), hs-CRP and Nt-pro-BNP (all $p<0.001$).

Data on dietary habits of 95 healthy controls and 637 RTR are shown in table 2. Caloric intake was significantly lower in RTR compared to healthy controls, which accounted for the other differences found in intake. Controlling for caloric intake, dietary habits were similar between RTR and controls, both regarding macro- and micronutrients. Vegetable intake did not differ between both groups (93±58 g/d vs 92±57; $p=0.9$)

Table 1 Characteristics of 107 healthy controls and 707 renal transplant recipients at the day of their visit to the outpatient clinic.

Characteristics	Healthy controls (n =107)	RTR (n = 707)	p diff
NOx excretion (nmol/24h)	1020 [796-1285]	588 [400-812]	<0.001
- Nitrite (nmol/24h)	0 [0-0]	0.77 [0.0-2.8]	<0.001
- Nitrate (nmol/24h)	1010 [795-1285]	565 [385-806]	<0.001
Demographics			
Age (years)	53± 10	53 ± 13	0.68
Male gender, n (%)	47	57	0.003
Waist Circumference, cm)	91.0 ± 10.5	98.6 ± 14.8	<0.001
BMI (kg/m^2)	26.5 ± 3.4	26.7 ± 4.8	0.73
BSA (m^2)	1.97 ± 0.19	1.94 ± 0.22	0.20
Current smoker, (%)	24	13	<0.001
Hemodynamic parameters			
Systolic BP, mmHg	125 ± 15	136 ± 18	<0.001
Diastolic BP, mmHg	75 ± 9	83 ± 11	<0.001
MAP, mmHg	92 ± 10	100 ± 12	<0.001
Heart Rate, bpm	67 ± 10	69 ± 12	0.07
Renal Function parameters			
Serum Creatinine, umol/L	73 [64-82]	125 [100-160]	<0.001
Creatinine Clearance (ml/min)	132 ± 41	66 ± 26	<0.001
eGFR, ml/min	93 ± 13	52 ± 20	<0.001
Albumin excretion	5.5 [3.1-8.3]	41.6 [10.6-179]	<0.001
Serum parameters			
Sodium, mmol/L	142 ± 1.9	141 ± 2.8	<0.001
Potassium, mmol/L	3.84 ± 0.26	3.98 ± 0.47	0.004
Chloride, mmol/L	106 ± 2.1	105 ± 3.5	0.006
Urea, mmol/L	5.7 [4.9-6.6]	9.6 [7.2-13.4]	<0.001
Albumin, mmol/L	45.2 ± 2.5	43.0 ± 3.0	<0.001
Cholesterol, mmol/L	5.3 [4.5-5.9]	5.0 [4.3-5.8]	0.14
Triglycerides, mmol/L	1.1 [0.8-1.6]	1.7 [1.3-2.3]	<0.001
HbA1c, %	5.55 ± 0.28	5.99 ± 0.83	<0.001
hsCRP,mg/L	1.2 [0.5-2.2]	1.6 [0.7-4.6]	<0.001
Nt-pro-BNP, ng/L	38 [20-65]	254 [109-614]	<0.001

Data are presented as mean ± SD, % or median [interquartile range]. Abbreviations: NOx combined urinary nitrite/nitrite excretion, BSA, body surface area, BMI, body mass index; BP blood pressure; MAP mean arterial pressure, eGFR, estimated Glomerular Filtration Rate; HbA1c glycated hemoglobine; hsCRP high-sensitive C-Reactive Protein; Nt-pro-BNP N-terminal pro-Brain Natriuretic Peptide. P for difference was tested by the independent t-test, kruskal-wallis test or χ^2-test.

Table 2 Dietary habits of 93 healthy controls and 637 renal transplant recipients based on food frequency questionnaires and urinary excretion rates.

Characteristics	Healthy controls (n = 93)	RTR (n = 637)	p diff
Dietary intake (nutrients)			
Calories, kcal	2309 ± 743	2175 ± 637	0.02
Protein, g/d (en%)	86 ± 24 (15.3)	82 ± 20 (15.5)	0.03 (0.24)
Animal protein, g/d (en%)	54 ± 18 (9.5)	52 ± 15 (9.8)	0.17 (0.11)
Vegetable protein, g/d (en%)	33 ± 11 (5.8)	31 ± 10 (5.7)	0.01 (0.29)
Total fat, g/d (en%)	94 ± 38 (36.1)	88 ± 34 (36.2)	0.06 (0.78)
Total carbohydrates, g/d (en%)	260 ± 88 (45.3)	249 ± 78 (45.8)	0.10 (0.27)
Calcium, g/d	1.11 ± 0.45	1.05 ± 0.38	0.05
Potassium, g/d	3.66 ± 1.00	3.53 ± 0.89	0.08
Magnesium, mg/d	355 ± 101	331 ± 90	0.002
Dietary intake (food groups)			
Meat, g/d	94 ± 42	95 ± 40	0.72
Milk, g/d	114 ± 110	117 ± 85	0.74
Cheese, g/d	42 ± 34	35 ± 28	0.01
Fruit, g/d	157 ± 114	152 ± 115	0.6
Vegetables, g/d	92 ± 57	93 ± 58	0.9
Potatoes, g/d	118 ± 69	130 ± 77	0.04
Bread, g/d	144 ± 69	133 ± 61	0.04
Urinary excretion, mmol/24h			
Creatinine	13.8 ± 4.2	11.8 ± 5.2	<0.001
Potassium (ratio with creatinine)	89 ± 29 (6.9)	73 ± 24 (6.5)	<0.001 (0.22)
Magnesium (ratio with creatinine)	5.3 ± 1.8 (0.42)	3.4 ± 1.6 (0.31)	<0.001 (<0.001)
Calcium (ratio with creatinine)	5.4 ± 2.4 (0.42)	2.9 ± 2.4 (0.26)	<0.001 (<0.001)
Sodium (ratio with creatinine)	212 ± 80 (16.2)	157 ± 62 (13.9)	<0.001 (<0.001)

Data are presented as mean ± SD or median [interquartile range]. Abbreviations: en% energy percentage. P for difference was tested by the independent t-test, kruskal-wallis test or χ^2-test.

As urinary NOx-excretion in RTR was significantly higher in men than in women (605 [448-829] nmol/24h vs 548 [350-771] nmol/24h resp.; p=0.006), characteristics are presented according to gender stratified tertiles of urinary NOx-excretion (*table 3*) with 57% of RTR being male in each tertile. Median NOx-excretion ranged from 326 [220-399] in the lowest tertile, through 588 [526-651] nmol/24h in the middle tertile to 916 [812-1072] nmol/24h in the highest tertile. With increasing NOx-excretion, RTR were significantly younger. In the lowest tertile of NOx-excretion, RTR had a significantly higher blood pressure despite more use of antihypertensive medication as compared to the highest tertile (MAP 101 mmHg vs 98 mmHg; p=0.04). Renal function, reflected by eGFR, increased significantly over tertiles of NOx-excretion (p<0.001) whereas albuminuria decreased (p=0.04). Compared with the highest tertile of urinary NOx-excretion, RTR in the lowest tertile had higher serum HbA1c (6.1±1.0 vs. 5.9±0.7; p=0.01), higher serum hs-CRP (1.8 [0.9-5.6] vs 1.3 [0.6-3.7]; p=0.05) and higher Nt-pro-BNP (440 [181-1086] vs 158 [69-368]; p<0.001).

The regression coefficients for the association of urinary NOx with various cardiovascular risk factors are given in *table 4*. After adjustment for potential confounders (model 4), urinary NOx-excretion was inversely associated with systolic blood pressure (st. β=-0.29; P=0.005), mean arterial pressure (st. β=-0.17; p=0.02), heart rate (st. β=-0.19; p=0.008), serum hs-CRP (st. β=-0.02; p=0.008), serum Nt-pro-BNP (st. β=-0.03; p<0.001), and HbA1c (st. β=-0.01; p=0.05). A significant positive association was found between NOx-excretion and estimated GFR (st. β=0.36; P=0.002). In the subgroup consisting of 201 RTR who underwent GFR measurement according to gold standard, we observed significant direct associations of urinary NOx-excretion with true GFR (st. β=0.24, p=0.002) and ERPF (st. β=0.28, p<0.001), independent of age, gender, BSA, smoking behavior, urinary sodium excretion and use of calcineurin inhibitors, diuretics and beta-blockers. No significant association was found between urinary NOx-excretion and filtration fraction.

Table 3 Characteristics of 707 renal transplant recipients across gender stratified tertiles of combined urinary nitrite/nitrate excretion (nmol/24h)

	Gender stratified tertiles of urinary NOx (umol/24h)			p-trend
	1 (n=235	2 (n=237	3 (n=235	
NOx excretion, nmol/24h	326 [220-399]	588 [526-651]	916 [812-1072]	
Demographics				
Age, y	54 ± 13	53 ± 13	52 ± 12	0.04
Waist, cm	99 ± 16	99 ± 14	98 ± 15	0.33
BMI, kg/m^2	26.9 ± 5.1	26.5 ± 4.6	26.6 ± 4.7	0.52
BSA, m^2	1.93 ± 0.24	1.95 ± 0.20	1.95 ± 0.22	0.36
Current smokers, %	12	12	14	0.63
Hemodynamic parameters				
Systolic BP, mmHg	138 ± 18	136 ±17	134 ± 17	0.005
Diastolic BP, mmHg	83 ± 12	83 ± 10	82 ± 11	0.31
MAP, mmHg	101 ± 13	101 ±11	99 ± 12	0.05
Heart Rate, bpm	55 ± 14	54 ± 13	52 ± 12	0.16
Medication use				
Hypertensives, %	94	85	86	0.006
RAS, %	54	42	48	0.17
Diuretics, %	50	40	33	<0.001
Beta-blocker	71	62	58	0.004
Antidiabetics, %	21	12	14	0.04
Statins, %	55	51	52	0.58
Proliferation inhibitor, %	80	83	86	0.14
Calcineurin inhibitor, %	70	53	50	<0.001
Renal Function parameters				
Time since Rtx, y	5.0 [1.4-11.0]	5.8 [2.0-12.6]	6.0 [3.0-12.3]	0.19
Serum Creatinine, umol/L	143 [110-188]	122 [97-154]	115 [98-144]	<0.001
Creatinine Clearance (ml/min)	54 ± 25	69 ± 25	75 ± 24	<0.001
eGFR, ml/min	46 ± 21	54 ±19	57 ± 18	<0.001
Albumin excretion	61 [13-223]	35 [11-178]	32 [9-138]	0.04
Serum parameters				
Sodium, mmol/L	141 ± 3	141 ± 3	141 ± 3	0.61
Albumin, mmol/L	42.4 ± 3.2	42.9 ± 2.8	43.6 ± 2.8	<0.001
Cholesterol, mmol/L	5.2 [4.4-6.0]	4.9 [4.3-5.8]	5.0 [4.3-5.7]	0.10
LDL-cholesterol, mmol/L	2.9 [2.4-3.6]	2.9 [2.3-3.4]	2.8 [2.3-3.6]	0.77
Triglycerides, mmol/L	1.78 [1.31-2.58]	1.67 [1.28-2.13]	1.56 [1.15-2.19]	0.29
HbA1c, %	6.1 ± 1.0	6.0 ± 0.8	5.9 ± 0.7	0.01
hsCRP,mg/L	1.8 [0.9-5.6]	1.6 [0.6-5.2]	1.3 [0.6-3.7]	0.05
Nt-pro-BNP, ng/L	440 [181-1086]	244 [97-502]	158 [69-368]	<0.001

Data are presented as mean ± SD, % or median [IQR]. Abbreviations: NOx combined urinary nitrate/nitrite excretion; BMI, body mass index; BSA, body surface area, BP, blood pressure; MAP mean arterial pressure; RAS, renin-angiotensin-system; RTx, renal transplantation; eGFR, estimated glomerular filtration rate; HbA1c glycated hemoglobin; hsCRP, high-sensitive CRP; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; PTH, parathyroid hormone; * P-trend was tested by either entering the median values within tertiles into the model as covariate, by χ^2-test or by Jonckheere-Terpstra test.

Tabel 4 Regression coefficients for the association of urinary NOx with several cardiovascular risk factors in 707 renal transplant recipients.

Cardiovascular risk factor	Model 1		Model 2		Model 3		Model 4	
	St. β	p	St. β	p	St. β	p	St. β	p
SBP, mmHg	-0.31	0.001	-0.28	0.004	-0.27	0.007	-0.29	0.005
DBP, mmHg	-0.12	0.05	-0.11	0.08	-0.11	0.09	-0.11	0.09
MAP, mmHg	-0.14	0.04	-0.17	0.01	-0.16	0.02	-0.17	0.02
Heart rate, bpm	-0.17	0.01	-0.21	0.003	-0.22	0.001	-0.19	0.008
hs-CRP*, mg/l	-0.03	0.001	-0.02	0.002	-0.02	0.003	-0.02	0.008
Nt-pro-BNP*, ng/l	-0.06	<0.001	-0.04	<0.001	-0.03	<0.001	-0.03	<0.001
HbA1c, %	-0.01	0.02	-0.01	0.002	-0.01	0.01	-0.01	0.05
Albuminuria*, g/24h	-0.01	0.21	0.01	0.47	0.001	0.95	-0.003	0.80
eGFR, ml/min	0.63	<0.001	#	#	0.42	<0.001	0.36	0.002
GFR**, ml/min	0.31	<0.001	#	#	0.23	0.002	0.24	0.002
ERPF**, ml/min	0.34	<0.001	#	#	0.28	<0.001	0.28	<0.001
FF**	0.14	0.07	#	#	0.06	0.41	0.07	0.39

Regression coefficients are given as standardized betas, i.e change of cardiovascular risk factor in SD, per SD increase of urinary NOx excretion. Abbreviations: BP, blood pressure; MAP mean arterial pressure; hs-CRP, high-sensitive CRP; Nt-pro-BNP, N-terminal pro-Brain Natriuretic Peptide; HbA1c glycated hemoglobin; eGFR, estimated glomerular filtration rate; ERPF effective renal plasma flow; FF filtration fraction. Model 1: adjusted for age (y), gender and BSA (m^2); Model 2: additionally adjusted for renal function (eGFR), Model 3: additionally adjusted for smoking behavior, urinary sodium excretion, medication use (calcineurin inhibitor, diuretic, beta-blocker), Model 4 additionally adjusted for 24h urinary magnesium excretion *Log-transformed for analyses **Analyses performed in a subgroup of 201 RTR

Discussion

The main finding of this study is the significant association between urinary NOx-excretion, reflecting systemic nitric oxide, and the extent of various cardiovascular risk factors in RTR. The associations remained significant after adjustment for age, gender, BSA, renal function, medication use and markers of vegetable intake. Second, we observed markedly lower urinary NOx-levels in RTR compared to healthy controls. Our

findings are consistent with our hypothesis that hampered NO is directly connected to endothelial dysfunction and increased cardiovascular risk long-term after renal transplantation.

Several mechanisms may underlie the decreased urinary NOx-excretion in RTR. These could either involve impaired synthesis, excessive oxidative degradation or both. NO is generated from L-arginine which is converted by nitric oxide synthase (NOS). In healthy subjects, the majority of the L-arginine in the circulation is synthesized by the kidney and the majority of L-arginine is eventually salvaged by reabsorption in the proximal tubules[29, 30]. Therefore, decreased NO production in RTR might have been caused by renal dysfunction which is in line with several studies that observed lower renal L-arginine release in patients with chronic kidney disease[31]. Previous studies suggested exogenous nitrate from vegetables to be a source of endogenously produced NO and consequently a potential target for treatment of cardiovascular disease [17, 18]. However, the observed differences in urinary NOx excretion between RTR and healthy controls was not explained by differences in dietary intake of exogenous NO-sources, since vegetable intake was similar between both groups. Within our transplant group, however, we observed higher urinary NOx-excretion in RTR with higher intake of vegetables, suggesting that exogenous nitrate donors might contribute to enhanced endogenous NO production. Yet, adjustment for markers of vegetable intake did not alter the associations between urinary NOx and various cardiovascular parameters in RTR, which suggests that systemic NO has a protective potential irrespective of the original source of the nitrogen.

Another factor potentially contributing to the decreased NO production in RTR is an increase in endogenous NOS inhibitors like asymmetric dimethylarginine (ADMA), a competitive inhibitor of NOS which has previously been found to be elevated in ESRD patients as well [32, 33]. Elevated plasma ADMA levels might be the consequence of a lack of the ADMA-degrading enzymes dimethylarginine dimethylaminohydrolase 1 and 2 (DDAH1 and 2) which in healthy subjects are highly expressed in the kidney and vasculature [34], both tissues being affected in RTR. Endothelial and renal dysfunction in RTR might therefore lead to decreased DDAH levels, subsequent increased ADMA levels leading to enhanced NOS-inhibition and decreased NO production, which paves the way to an increased susceptibility to cardiovascular disease and progression of renal dysfunction, a hypothesis that is supported by several previous studies as well as by our findings [35]. Another potential factor underlying the lower urinary NOx excretion in RTR compared to healthy controls might be an excessive degradation due to the continuous state of oxidative stress, characterized by an extensive production of oxygen radicals (O_2^-) which, together with NO, leads to formation of peroxynitrite and subsequently nitrotyrosin, causing injury to proteins and tissues [36, 37].

Since RTR are prone to cardiovascular and inflammatory events, possibly due to endothelial dysfunction, it could very well be a shortage of endothelial NOS (eNOS) playing a cardinal role in these pathofysiological processes. However, whether it is primarily endothelial dysfunction that subsequently leads to decreased NO production or vice versa, and whether NO should be considered a biomarker or a causative factor in the development of CVD in RTR remains to be solved. Nevertheless, a decline in NO production, either primary or secondary, ultimately leads to pathological vascular conditions due to loss of the protective potential of NO. As a consequence, inflammation, enhanced vascular constriction and platelet aggregation might occur, all of these processes in turn affecting the endothelium and its NO synthesizing potential indicating that a reduction in NO is at least involved in the progression of CVD in RTR. In line, Albrecht *et al.* demonstrated a combination of decreased urinary NOx excretion and loss of glomerular eNOS expression in biopsy specimens diagnosed as either acute rejection or chronic renal transplant failure [12, 13]. Since endothelial NO production has important functions with respect to inhibition of platelet aggregation and vascular smooth muscle cell proliferation, the diminished activity of endothelial NO production during both acute rejection and chronic renal transplant failure, might underlie the high prevalence of inflammation, cardiovascular diseases and mortality among RTR. Our findings of highly significant associations of urinary NOx excretion with blood pressure, heart rate, NT-pro-BNP, HbA1c and hs-CRP, all known to be associated with vascular conditions, make NO production and endothelial function attractive central pathophysiological factors in the treatment of post-transplant CVD and mortality. Therefore, modulation of the NO system by restoring eNOS expression or endothelial NO availability could receive more attention in the renal transplantation setting.

We have to acknowledge that the present study has some limitations. Its cross-sectional design makes causality hard to prove. Also, since the study population almost entirely consisted of Caucasian RTR, the applicability of our results to racially diverse RTR populations remains limited. Also, our study is a single-center study which might limit generalizability. On the other hand, the large size of the study population involved, the large diversity in times since transplantation, the use of 24 hour urine samples for inference of NO production and the extensive data collection, including dietary intake, allowing for adjustment for many potential confounders, renders our finding of a significant association between hampered NO production and the extent of cardiovascular risk in RTR reliable and robust.

Large, long-term intervention studies on influencing endothelial NO synthesis in stable human renal transplant recipients are lacking. New studies in RTR should focus on either administration of NO substrates, like L-arginine, or NO donors like nitrate. Although vegetable intake in RTR was similar compared with healthy controls, we can not

exclude a potentially beneficial long-term effect of increased vegetable intake in RTR. A promising new player in the field of NO donors might also be nitrite. Recently, Kelpke et al observed the transplanted kidney to be protected against ischemic/reperfusion injury after administration of sodium nitrite ultimately leading to improved post-transplant kidney function.

In conclusion, post-transplant conditions are associated with a markedly increased risk for cardiovascular events. The observed tapering of NO production following renal transplantation may be representative for endothelial damage and by itself play a role in the progression of structural or functional vascular changes. These results prompt for the evaluation of long-term administration of NO donors for treatment or even prevention of post-transplantation cardiovascular disease.

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7

Vitamin K Intake and Plasma Desphospho-Uncarboxylated Matrix Gla-Protein Levels in Renal Transplant Recipients

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Abstract

Background Vitamin K is essential for activation of γ-carboxyglutamate (Gla)-proteins including the vascular calcification inhibitor matrix Gla-protein (MGP). Insufficient vitamin K intake leads to production of uncarboxylated, mostly inactive proteins and contributes to an increased cardiovascular risk. In kidney transplant recipients, cardiovascular risk is high but vitamin K intake and status have not been defined. We investigated dietary vitamin K intake, vascular vitamin K status and its determinants in kidney transplant recipients.

Methods We estimated vitamin K intake in a cohort of kidney transplant recipients (n=60) with stable renal function (creatinine clearance 61 [42-77] (median [interquartile range]) ml/min), who were 75 [35-188] months after transplantation, using three-day food records and food frequency questionnaires. Vascular vitamin K status was assessed by measuring plasma desphospho-uncarboxylated MGP (dp-ucMGP).

Results Total vitamin K intake was below the recommended level in 50% of patients. Lower vitamin K intake was associated with less consumption of green vegetables (33 vs 40 g/d, p=0.06) and increased dp-ucMGP levels (621 vs 852 pmol/L, p<0.05). Accordingly, dp-ucMGP levels were elevated (>500 pmol/L) in 80% of patients. Multivariate regression identified creatinine clearance, coumarin use, body mass index, high sensitivity-CRP and sodium excretion as independent determinants of dp-ucMGP levels.

Conclusion In a considerable part of the kidney transplant population, vitamin K intake is too low for maximal carboxylation of vascular MGP. The high dp-ucMGP levels may result in an increased risk for arterial calcification. Whether increasing vitamin K intake may have health benefits for kidney transplant recipients should be addressed by future studies.

Introduction

Vitamin K deficiency is increasingly recognized as a risk factor for cardiovascular morbidity and mortality in renal patients [1, 2]. Vitamin K refers to a set of different fat-soluble vitamins occurring as phylloquinone (vitamin K1) or a series of vitamins commonly termed menaquinones (vitamin K2). The main sources of vitamin K1, the most prominent form of vitamin K in the Western diet, are green vegetables and dairy products. Vitamin K2 comes from fermented food such as cheese and curd [3, 4], and is mainly considered to be produced by bacterial flora in the intestinal tract. Both vitamers serve as cofactors for modifying glutamate into gamma-carboxylated glutamate (Gla) residues in biological active proteins, including matrix Gla protein (MGP) [5]. In addition, vitamin K2 is a membrane-bound electron carrier in mitochondria [6]. MGP, which is synthesized by vascular smooth muscle cells and chondrocytes, is an important inhibitor of vascular calcification [7]. Poor vitamin K status due to poor intake or the use of vitamin K antagonists results in high uncarboxylated MGP (ucMGP) levels and is associated with vascular calcification, both in populations with and without renal disease [7, 8]. The plasma desphospho-ucMGP (dp-ucMGP) fraction is considered a marker for vascular vitamin K status [9, 10]. The actual contribution of dietary vitamin K intake to the vascular vitamin K status is not yet known, supplementation with menaquinone-7 (one of the K2 vitamins) may reduce dp-ucMGP levels in hemodialysis patients [11].

It has recently been demonstrated that the majority of hemodialysis patients have vitamin K deficiency as reflected by high dp-ucMGP levels [1], as well as low vitamin K intake [12]. Their low vitamin K intake may derive from the dietary regimen generally prescribed to hemodialysis patients, which includes restriction of sodium and potassium intake. Therefore dialysis patients limit their intake of mainly green vegetables and cheeses, i.e food products that are rich in vitamin K1 and K2, respectively. Factors other than dietary intake such as compromised renal function may contribute to the vitamin K status as well[13]. The vitamin K insufficiency present in the majority of hemodialysis patients may contribute to their strongly increased risk for arterial calcification development[14-16].

Although cardiovascular morbidity and mortality after kidney transplantation are lower compared to any of the dialysis modalities, the risks are considerably higher than those in the general population [17]. Whether vitamin K insufficiency is also common in kidney transplant recipients is unknown, but several factors associated with reduced vitamin K status such as impaired renal function remain present in many patients after kidney transplantation, and thus could affect vitamin K status. A recent study documented that kidney transplant recipients consume less sodium and potassium than the general population [18], but their dietary vitamin K intake has not been documented.

The objective of this study was to determine the intake of vitamin K1, vitamin K2 and total vitamin K and vascular vitamin K status, by measuring desphospho-ucMGP (dp-ucMGP) levels, in kidney transplant recipients. Furthermore, we aimed to identify dietary factors that are associated with vitamin K status in this patient group.

Materials and methods

Ethics statement

The study was approved by the medical ethics committee of the University of Groningen (METc 2008/186), and all participants provided written informed consent. All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

Study population

No formal power calculation was performed for this explorative observational study. Kidney transplant recipients attending the outpatient clinic of the University Medical Center Groningen were recruited. Patients with a minimum age of 18 years and able to return completed food records were eligible, irrespective of gender, the underlying primary renal disease, the presence of cardiovascular disease, diabetes mellitus, or other traditional cardiovascular risk factors. Exclusion criteria were known malignancy, abnormal liver function tests, history of gastrointestinal disease or metabolic disease, or active infection. In total, 60 patients with a functioning kidney graft for at least one year were enrolled.

Standard immunosuppressive therapy was as follows: from 1968 until 1989 prednisolone (10 mg/day) and azathioprine (100 mg/day). From January 1989 until February 1993 ciclosporin standard formulation (Sandimmune, Novartis; 10 mg/kg; trough levels of 175–200 mg/l in first 3 months, 150 mg/l between 3 and 12 months post-transplant and 100 mg/l thereafter) combined with prednisolone (starting with 20 mg/day, rapidly tapered to 10 mg/day). From March 1993 until May 1996 ciclosporin microemulsion (Neoral, Novartis Pharma b.v., Arnhem, The Netherlands; 10 mg/kg; trough levels idem) and prednisolone. From May 1996 to date mycophenolate mofetil (MMF) (Cellcept, Roche b.v., Woerden, The Netherlands; 2 g/day) was added. Current medication was extracted from the medical records.

Vitamin K intake

Dietary intake was assessed using a dietary diary that was kept during three consecutive days in advance of the patients' visit to the outpatient clinic. All patients adhered to

their normal dietary habits. A trained researcher checked whether diaries were filled out properly, and if necessary additional information was obtained about unusual or missing reports. Intake was recorded in household measurements and standard portion sizes. For calculations of the intakes of total energy and nutrients, we used the Food Calculation System (BAS nutrition software 2004, Arnhem, The Netherlands) in which Dutch food composition database NEVO 2006 was included[19]. Concentrations of vitamin K1 and K2 (MK-4 through MK-10) of 260 foods have been added to the NEVO (2006) food database, as described previously[15]. Dietary intake of total vitamin K, vitamin K1 and vitamin K2 from three consecutive days were averaged and used for analysis.
The U.S. Dietary Reference Intake for an adequate intake of vitamin K for adult men is 120 micrograms/day and for adult women 90 micrograms/day[20].

Habitual dietary intake
Additional information on dietary intake was obtained using a semi quantitative food frequency questionnaire (FFQ) that inquired about intake of 177 food items. For each item, the frequency was recorded in times per day, week, or month. The number of servings per frequency was expressed in natural units or household measures. The questionnaire was self-administered and filled out at home. Before participation in this study, all patients were carefully instructed by a trained researcher on how to complete the three day dietary diaries. In addition, similar written instructions were provided at the first page of the diary. The FFQs were checked for completeness and inconsistent answers were verified with the patients. Additionally, all participants were instructed to collect a 24 hour urine sample according to a strict protocol. In addition, excretion of several urinary components was measured to infer dietary intake of additional dietary nutrients like sodium and potassium.

Vitamin K status
Blood was drawn after an 8-12h overnight fasting period in the morning after completion of the dietary diary. Vitamin K status was assessed by measuring dp-ucMGP. Before serum preparation, blood was kept for 20 min at room temperature. Plasma and serum were prepared by standard centrifugation and stored at -80 °C until testing.
Circulating dp-ucMGP levels were determined in citrated plasma using a dual-antibody ELISA (VitaK BV Maastricht The Netherlands). In this assay, the capture antibody is directed against the non-phosphorylated MGP sequence 3-15 and the detection antibody against the ucMGP sequence 35-49 or the carboxylated MGP sequence 35-54, respectively, as described previously [2, 13]. Vitamin K insufficiency was defined as dp-ucMGP levels of > 500 pmol/L [13].

Additional parameters

Renal function was assessed by calculating 24h urinary creatinine clearance (ml/min). Serum creatinine levels were determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Plasma and urinary concentrations of electrolytes and urea were measured using routine clinical laboratory methods, as were serum cholesterol, N-terminal-pro-brain natriuretic peptide (NT pro-BNP), high sensitivity CRP. Information on patients' health status, medical history and medication use was obtained from patient records. Questionnaires were used to obtain information on smoking behavior. Participants were classified as current smokers, former smokers, or never smokers. Body weight and height were measured while participants wore indoor clothing without shoes. Body Mass Index (BMI) was calculated as weight divided by height squared (kg/m^2).

Statistical analysis

Anthropometric, clinical and laboratory parameters were compared between subjects with normal versus poor vitamin K intake using the Mann-Whitney non-parametric test or chi square test where appropriate. Similar analyses were performed to compare the characteristics of patients with normal versus elevated plasma dp-ucMGP levels.

Subsequently, linear regression analysis was performed to identify independent determinants of plasma dp-ucMGP levels, according to a gradual modeling approach. We started with a model containing age, gender, and renal function. Subsequently, parameters that significantly differed between subjects with normal versus poor vitamin K status were subsequently put into the regression model. If significant, the parameter remained in the model; if not significant, the parameter was removed from the model. This strategy was chosen to avoid a too large number of degrees of freedom when all possible covariates were put into the model at once. Non-normally distributed variables were transformed to the natural log before entering into the regression model.

Data are presented as mean±standard deviation or median (interquartile range), depending on their distribution (normal or non-normal, respectively), or percentages. A two-sided p-value <0.05 was considered statistically significant Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Corp. Chicago, IL).

Results

The study population consisted of 30 male and 30 female kidney transplant recipients, who were at median 75 [interquartile range 35-188] months after kidney transplantation. Creatinine clearance was 61 ml/min [42-77 ml/min]. A detailed overview of baseline parameters is presented in Table 1.

As shown in Table 1, total vitamin K intake was below the recommended level in 50% of all subjects, both in men and women. Lower vitamin K intake was associated with increased dp-ucMGP levels reflecting vascular vitamin K insufficiency. Patients with a low total vitamin K intake were more likely to have been on dialysis prior to transplantation and had a trend towards longer dialysis vintage (p=0.05). Their diet contained more sodium, as reflected by an increased 24h-urinary sodium excretion (p=0.001), and less dietary fiber (p=0.02), with similar caloric intake between both groups. Dietary protein intake was positively associated with 24h-urinary urea excretion (r=0.566, p<0.001), and dietary potassium intake was positively associated with 24h-potassium excretion (r=0.468, p<0.001), suggesting that dietary diary information was representative of the actual intake as reflected by the 24h-urinary excretion. Analysis of the intake of specific dietary products (Table 2) from food frequency questionnaires revealed that patients with poor vitamin K intake tended to eat less green vegetables (p=0.056), the most prominent dietary source of vitamin K1, as compared to subjects with normal vitamin K intake. Conversely, patients with poor vitamin K intake tended to use more milk (p=0.06). A more detailed overview of dietary intake for both groups is provided in Supplementary Table S1.

Eighty percent of kidney transplant recipients had elevated dp-ucMGP levels, both in males (24/30) and in females (24/30). Vitamin K status was lower in patients on calcineurin inhibitors than in patients not using these drugs (dp-ucMGP levels 855 [590-1350] vs 616 [472-888] pmol/L, p=0.006). On the contrary, dp-ucMGP levels were lower in subjects on azathioprine or cellcept (721 [504-917] vs 1073 [870-1733] pmol/L, p=0.008) compared with patients not on these drugs. Separate analyses for both drugs revealed that patients on mycophenolate mofetil had lower dp-ucMGP levels as compared to those not on mycophenolate mofetil (591 [479-897] vs 917 [741-1412] pmol/L, p=0.002), but the use of azathioprine did not influence dp-ucMGP levels (882 [703-1088] vs 726 [491-1091] pmol/L, p=0.14). In patients using vitamin K antagonists (*n=6*), dp-ucMGP levels were higher than in patients not on vitamin K antagonists (*n=54*) with 725 pmol/L (516-923) vs 2079 pmol/L (1.658-2.272), p<0.001). Patients with elevated dp-ucMGP levels were more likely to have been on dialysis (96% vs 75%, p=0.02) and tended to have had longer dialysis vintage (41 (17-61) vs 18 (1-30) months, p=0.05) compared to patients with normal dp-ucMGP levels. Furthermore, patients with increased dp-ucMGP also had lower creatinine clearance (60 (45-75) vs 78 (52-83) ml/min, p=0.04), higher levels of albuminuria (119 (62-304) vs 51 (30-75), p=0.003, and tended to have higher NTproBNP levels (262 (118-825) vs 147 (70-355), p=0.054) compared to patients with normal dp-ucMGP. All other variables (listed in Table 1) were not significantly different between vitamin K sufficient and insufficient patients.

Table 1 Patient characteristics according to total vitamin K intake.

	Total Cohort	Normal Vitamin K Intake	Poor Vitamin K Intake	p
Gender (male), n (%)	30 (50%)	15 (50%)	15 (50%)	0.99
Age, years	55±10	55±11	56±10	0.84
BMI, kg/m^2	26.2±4.6	23.8± 3.8	26.2± 4.4	0.03
SBP, mmHg	135±17	136±16	133±17	0.45
Current or previous smoking	31 (52%)	20 (67%)	11 (37%)	0.02
Presumed cause of ESRD, n (%)				
Glomerulonephritis/vasculitis	10 (17%)	5 (17%)	5 (17%)	0.99
Membranous glomerulopathy/ FSGS	1 (2%)	1 (3%)	0 (0%)	0.33
Vascular disease/ hypertension	5 (8%)	4 (13%)	1 (3%)	0.17
IgA nephropathy	5 (8%)	0 (0%)	5 (17%)	0.02
ADPKD and MCKD	17 (28%)	9 (30%)	8 (27%)	0.78
Diabetic nephropathy	1 (2%)	1 (3%)	0 (0%)	0.33
Urological origin	7 (12%)	1 (3%)	6 (20%)	0.05
Other/unknown	14 (23%)	9 (30%)	5 (17%)	0.23
Transplant characteristics				
Time since transplantation, months	75 (35-188)	65 (34-179)	93 (33-199)	0.44
Type of last transplant				
Living donor	16 (26.7%)	10 (33%)	6 (20%)	0.25
Cadaveric donor	44 (73.3%)	20 (67%)	24 (80%)	0.25
Transplants received				
Kidney only	57 (95%)	28 (93%)	29 (97%)	0.57
Simultaneous liver-kidney	2 (3.3%)	1 (3%)	1 (3%)	0.98
Simultaneous pancreas-kidney	1 (1.7%)	1 (3%)	0 (0%)	0.33
Dialyis prior to transplantation	55 (92%)	25 (83%)	30 (100%)	0.02
Dialysis vintage, months	31 (17-59)	28 (5-49)	45 (23-67)	0.05
Diabetes	8 (13%)	4 (13%)	4 (13%)	0.97
Hypertension	56 (93%)	30 (100%)	26 (87%)	0.04
Hypercholesterolemia	43 (72%)	20 (67%)	34 (77%)	0.40
Previous CVD	9 (15%)	5 (17%)	4 (13%)	0.73
Current medication use				
Immunosuppressive drugs				
Prednisolon	59 (98%)	30 (100%)	29 (97%)	0.33
Calcineurin inhibitor	34 (57%)	14 (47%)	20 (67%)	0.12
Ciclosporin	26 (76%)	10 (71%)	16 (80%)	0.12
Tacrolimus	8 (24%)	4 (29%)	4 (20%)	0.99
Mycophenolate mofetil	39 (65%)	21 (70%)	18 (60%)	0.43
Azathioprine	12 (20%)	7 (23%)	5 (17%)	0.53
mTOR inhibitor	2 (3%)	2 (7%)	0 (0%)	0.16
Statins	36 (60%)	17 (57%)	19 (63%)	0.61
Diuretics	16 (27%)	6 (20%)	10 (33%)	0.25
β-Blockers	40 (67%)	20 (67%)	20 (67%)	0.99
RAS-inhibitors	28 (47%)	11 (37%)	16 (53%)	0.20
Calcium channel blockers	12 (20%)	6 (20%)	6 (20%)	0.99
Coumarin	6 (10%)	1 (3%)	5 (17%)	0.09
Aspirin	15 (25%)	9 (30%)	6 (20%)	0.38

Serum hsCRP, mg/dL	0.11 (0.04-0.28)	0.11 (0.04-0.15)	0.15 (0.05-0.49)	0.16
Serum creatinine, mg/dL	1.5 (1.2-1.8)	1.4 (1.3-1.8)	1.5 (1.2-1.9)	0.91
Serum uric acid, mg/dL	7.2 (5.8-8.4)	6.7 (5.7-8.0)	7.5 (6.1-8.6)	0.20
Serum total cholesterol, mg/dL	189 (170-228)	189 (174-224)	197 (162-232)	0.95
Serum NT pro-BNP, pg/mL	245 (113-719)	151 (106-476)	281 (130-863)	0.27
dp-ucMGP, pmol/L	753 (543-1091)	621 (481-927)	852 (620-1350)	0.04
Urinary sodium, mmol/24h	144±60	115± 41	153±52	0.001
Urinary urea nitrogen, mg/24h	11 (9-12)	11 (9-13)	11 (9-12)	0.66
Urinary potassium, mmol/24h	72±23	75± 22	71±21	0.13
Urinary albumin, mg/24h	94 (48-247)	63 (36-246)	120 (73-248)	0.11
Creatinine clearance, mL/min	64±26	59±19	63± 24	0.69
Dietary Intake (Recomm. Intake[34])				
Vitamin K1, µg/day	73 (36-153)	150 (110-270)	36 (23-60)	<0.001
Vitamin K2, µg/day	14 (5.3-25.3)	14 (4.7-29)	14 (5.8-23)	0.62
Total vitamin K, µg/day (M:120, F:90)	90 (53-175)	171 (138-283)	54 (45-77)	<0.001
Total kCal/day (M:2300, F:1800)	2204±533	2222±538	2211±477	0.80
Total protein, g/day (10-35%[a])	78.2 (67.8-86.1)	85±21	81±14	0.29
Vegetable protein	28.7 (24.8-36.8)	33±8	31±8	0.14
Animal protein	47.9 (40.5-54.0)	52±18	50±11	0.53
Carbohydrates, g/day (45-65%[a])	183.9 (2.4-259.8)	269±83	259±59	0.19
Total fat, g/day (20-35%[a])	73.0 (58.0-91.8)	85±25	88±26	0.71
Saturated fatty acids	27.0 (20.7-33.1)	30±11	31±9	0.46
Mono-unsaturated fatty acids	22.1 (18.2-29.4)	29±9	29±9	0.92
Poly-unsaturated fatty acids	14.2 (11.7-19.4)	19±6	20±8	0.83
Dietary fiber, g/day (M: 28, F: 22)	19.9 (0.04-26.6)	22.3 (0.4-29.2)	12.8 (0.02-21.9)	0.02
Potassium, mg/day (4700)	2986 (131-3881)	3811±745	3480±620	0.11
Alcohol, g/day (M<20, F<10)	10.8 (0-207.2)	3.2 (0-181.9)	21.7 (0.1-249.5)	0.05

Normal intake was defined as ≥120 µg/day (males; M) or ≥90 µg/day (females, F)[20]. P value for comparison of subjects with normal vs poor vitamin K intake was obtained with the Mann Whitney U test. Nutritional goals apply to ages 51 years and older. [a] Percentage of total energy intake
Abbreviations: BMI, body mass index; SBP, systolic blood pressure; ADPKD, autosomal dominant polycystic kidney disease; MCKD, medullary cystic kidney disease; CVD, cardiovascular disease; mTOR, mammalian target of rapamycin; hsCRP, high-sensitivity C-reactive protein; NT-proBNP, N terminal-pro brain natriuretic peptide; MGP, matrix gla protein.

Table 2 Habitual dietary intake per vitamin K-containing food component assessed by food frequency questionnaires

Dietary Component	Normal vitamin K intake (*n=30*)	Poor vitamin K intake (*n=30*)	p
Green Vegetables, g/d	39.8 [31.2-55.8]	32.6 [21.3-46.8]	0.06
Broccoli, cauliflower, g/d	14.9 [12.0-20.9]	11.6 [8.3-17.5]	0.05
Lettuce, spinach, endive, g/d	24.4 [16.4-36.8]	19.4 [6.9-30.5]	0.08
Cheese, g/d	27.4 [20.8-44.7]	27.2 [20.0-39.8]	0.47
Butter, g/d	34.6 [18.0-52.6]	25.6 [18.0-47.6]	0.47
Oil, g/d	0.62 [0.0-4.8]	0 [0.0-1.9]	0.19
Meat, g/d	32 [14.3-34.4]	26.9 [19-39]	0.42
Milk, g/d	146 [52-300]	259 [159-313]	0.06

Normal intake was defined as ≥120 μg/day (males; M) or ≥90 μg/day (females, F)[20] Data are expressed as median [interquartile range]

Upon multivariate analysis creatinine clearance, coumarin use, body mass index, 24h-urinary sodium excretion (borderline) and high sensitivity CRP (borderline) were independent determinants of the vascular vitamin K status (Table 3). NT-proBNP levels and the type of immunosuppressive therapy were not significant determinants of vitamin K status in the multivariate model; their significance was lost when creatinine clearance was present in the model.

Table 3 Determinants of vascular vitamin K status (plasma dp-ucMGP)

Model	St. β	p
Model 1: Age, gender, creatinine clearance		
Age	0.17	0.21
Gender	-0.17	0.22
Creatinine clearance	-0.43	0.001
Model 2: Model 1 + coumarin use		
Creatinine clearance	-0.32	0.002
Coumarin use (0=no, 1=yes)	0.52	<0.001
Model 3: Model 2 + albuminuria		
Creatinine clearance	-0.37	0.01
Coumarin use (0=no, 1=yes)	0.36	0.02
Albuminuria	0.04	0.79
Model 4: Model 3 + pre-transplant dialysis		
Creatinine clearance	-0.33	0.002
Coumarin use (0=no, 1=yes)	0.50	<0.001
Pre-transplant dialysis	0.18	0.07
Model 5: Model 4 + high sensitivity CRP		
Creatinine clearance	-0.37	0.001
Coumarin use (0=no, 1=yes)	0.32	0.009
Pre-transplant dialysis (0=no, 1=yes)	0.18	0.09
High sensitivity CRP	0.27	0.03
Model 6: Model 5 + BMI		
Creatinine clearance	-0.45	<0.001
Coumarin use (0=no, 1=yes)	0.36	0.001
Pre-transplant dialysis (0=no, 1=yes)	0.16	0.09
High sensitivity CRP	0.19	0.08
Body mass index	0.37	0.001
Model 7: Model 6 + 24h-urinary sodium excretion		
Creatinine clearance	-0.51	<0.001
Coumarin use (0=no, 1=yes)	0.31	0.005
Pre-transplant dialysis (0=no, 1=yes)	0.09	0.36
High sensitivity CRP	0.18	0.09
Body mass index	0.35	0.001
24h-urinary sodium excretion	0.22	0.05

Determinants of dp-ucMGP levels, reflecting vascular vitamin K status, analyzed by stepwise multivariate linear regression analysis. Abbreviations: dp-ucMGP, desphospho-uncarboxylated matrix gla protein; CRP, C-reactive protein; Non-parametric variables (creatinine clearance, albuminuria, high sensitivity CRP) were Ln-transformed before entering into the model.

Discussion

The current study shows for the first time that both insufficient vitamin K intake and vascular vitamin K insufficiency (deduced from circulating dp-ucMGP levels) are very common in a population of stable kidney transplant recipients. Vitamin K deficiency in kidney transplant recipients has been reported previously by means of a coagulopathy responsive to vitamin K treatment in case series [21]. Mazzaferro et al recently argued that total MGP levels in kidney transplant recipients were close to normal [22]; however their study did not differentiate between carboxylated and uncarboxylated MGP which is important given the role for vitamin K in MGP carboxylation. When dp-ucMGP, an appropriate marker of vascular vitamin K status [2-4], was taken into account specifically in our study, we found clear indications of vascular vitamin K insufficiency in the majority of kidney transplant recipients.

Dietary recommendations for an adequate intake of vitamin K are based on the hepatic vitamin K1 requirement to for coagulation factor synthesis [20]. It does not account for vitamin K2 requirement to inhibit vascular calcification. The actual necessary amount of vitamin K intake based on the role of extra-hepatic vitamin K-dependent proteins is still unknown but studies suggest that dietary recommendations for vitamin K are too low to ensure full carboxylation of MGP [8]. Indeed, in our study even the subjects with adequate vitamin K intake according to U.S. guidelines [20] still had median dp-ucMGP levels above the recommended level of 500 pmol/L, suggesting vascular vitamin K insufficiency. This suggests that either kidney transplant recipients should be recommended to increase their dietary vitamin K intake beyond amounts recommended to the general population, or these patients should be supplemented with extra vitamin K.

Whether for vascular vitamin K status the intake of vitamin K2 is superior to vitamin K1 is uncertain, but the intake of vitamin K2 appears to be more important than vitamin K1 to prevent coronary heart disease [14]. Furthermore, in a recent pilot study in hemodialysis patients, a reduction of dp-ucMGP was dose-dependently achieved by treatment with vitamin K2 [11]. This can be mechanistically explained by the fact that the main transporters of vitamin K1 are triglyceride-rich lipoproteins that are retained by the liver and serve as a cofactor for proteins involved in coagulation. The vitamin accumulation and use in extrahepatic tissues such as the vascular wall, is low. Vitamin K2 on the other hand is transported not only by triglyceride-rich lipoproteins, but also by low density lipoproteins, the main carrier system to extrahepatic tissues [23]. In our study, subjects with poor vitamin K intake had increased dp-ucMGP levels (lower vascular vitamin K status) compared to those with normal vitamin K intake. On the other hand, dietary vitamin K intake (neither vitamin K1 nor K2 or total vitamin K) was not an independent determinant of dp-ucMGP levels upon multivariate analysis. This suggests that either other factors

such as renal function, together with coumarin use as an iatrogenic factor, are more important determinants of vascular vitamin K status in this population. Dietary intake of vitamin K2 intake may also be an inappropriate reflection of the actual amount of vitamin K2 generated by intestinal micro-organisms. The composition of the intestinal flora, and importantly the presence of Bacteroides species, the main producers of vitamin K2 [24], is influenced by dietary factors including fibers [25]. The subjects with poor vitamin K intake in our population ate significantly less fiber than those with normal vitamin K intake; this may have affected intestinal vitamin K2 production.

The observation that vascular vitamin K insufficiency was more common than may be expected by vitamin K intake alone could be explained for a considerable part by the contribution of renal function impairment. Our data confirm the previously known relationship between renal function[22, 26] and dp-ucMGP levels and the influence of vitamin K antagonists[11]. Whether, as in hemodialysis patients[11], vitamin K2 supplementation may also reduce dp-ucMGP levels in kidney transplant recipients, especially those with compromised renal function, remains to be addressed in prospective trials. Our finding that vascular vitamin K status was associated with body mass index in multivariate analysis is in line with previous studies linking vitamin K status with parameters of glucose metabolism and atherosclerosis [27]. The borderline significant association between dp-ucMGP levels and 24h-urine sodium excretion suggests that high dietary sodium intake may negative affect vitamin K metabolism. Although vitamin K deficiency [1, 2] and high sodium intake [28] have both been associated with adverse cardiovascular outcomes, their possible interactions have not been addressed.

We found a trend towards an inverse relationship between vitamin K status and low-grade inflammation. Cell culture studies have shown anti-inflammatory effects of vitamin K in lipopolysaccharide-treated fibroblasts through inhibition of interleukin-6 [29]. In animals, a vitamin K-deficient diet enhanced the expression of inflammatory genes, which was reversed by vitamin K1-supplemented diets; furthermore the supplemented diet suppressed the inflammatory response induced by lipopolysaccharide [30]. Recently, both plasma vitamin K status and intake were inversely related to inflammatory markers in a human general population cohort [31].

The reduced vascular vitamin K status in patients on calcineurin inhibitors is in line with the increased risk of cardiovascular complications in patients on these drugs, particularly ciclosporin [32]. On the other hand, in our multivariate analysis of determinants of vitamin K status, none of the immunosuppressive drugs remained in the model after co-adjustment for creatinine clearance, suggesting that the differences in vitamin K status can be explained by differences in creatinine clearance. A recent report suggested that MGP levels were higher in patients on mTOR inhibitors [33]; unfortunately our cohort contained only two patients using this class of drugs.

Our study has the limitation of being a relatively small single center study in Caucasian patients only, so the generalizability of our findings will require support by studies in other populations. The limited sample size may have influenced the results of multivariate regression analysis, e.g. regarding the role of immunosuppressive regimens as determinants of vitamin K status. Furthermore, although dp-ucMGP levels have been associated with cardiovascular morbidity and mortality in the chronic kidney disease population [1,2], these associations have not yet been established for the kidney transplant population.

In conclusion, we found that elevated dp-ucMGP levels, reflecting vascular vitamin K insufficiency, is common in kidney transplant recipients. Poor vitamin K intake is common in renal transplant recipients, and our data suggest that other factors including renal function may contribute to poor vascular vitamin K status as well. Correction of vitamin K status might be clinically relevant, given the known associations of vascular vitamin K deficiency with cardiovascular outcomes. Whether this can be achieved by relatively simple dietary measures in kidney transplant recipients should be addressed in future prospective studies.

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8

Summary, Discussion and Future Perspectives

Summary

Worldwide, the prevalence and incidence of end stage renal disease (ESRD) is increasing steadily[1, 2], most likely attributable to increasing occurrence of lifestyle associated diseases such as diabetes type 2 and hypertension [3-6]. These disorders together account for more than 60% of the incident ESRD cases nowadays. Also in the Netherlands these trends are observed. Last year, the total number of patients with treated ESRD grew with about 1,900 and reached almost 16,000 patients, which is 1 patient per 1000 inhabitants[7]. Of these patients, 60 percent, or 10,000 ESRD patients, have a functioning renal graft nowadays. In barely ten years, the number of ESRD patients with a functioning graft has doubled and the prevalence continues to increase impressively. These rising numbers can be attributed not only to the increase in the number of patients who receive a renal graft but also to improved survival after renal transplantation[8, 9]. Indeed, graft survival over the first year post-transplantation has increased remarkably from 40 percent in the 1970's to over 90 percent in present days for recipients of deceased donor kidneys [2]. Unfortunately, long term graft and also patient survival have hardly improved over the last decades. Renal transplant recipients are confronted with frequent occurrence of cardiovascular morbidity, which is the leading cause of graft loss and mortality[10, 11] . Also, patients are commonly afflicted by metabolic disorders due to impaired renal function and adverse effects of long-term drug use[12-14]. Therefore, attention should be focused on the search for tools to help improve long-term health after transplantation. Since lifestyle modifications, including dietary habits, have been proven to play a role in health status in the general population and in CKD patients [15-20], we hypothesized that nutritional factors could provide new targets for intervention in renal transplant recipients as well to improve long-term morbidity and mortality.

The aim of this thesis was to document the nutritional intake in a large cohort consisting of stable renal transplant recipients with a functioning graft for at least one year and to compare their dietary habits with those in a healthy reference group. Second, we aimed to investigate the associations of several nutritional factors with cardiovascular, metabolic and renal risk profiles long-term after renal transplantation to identify potential targets for intervention to help prevent morbidity and mortality in renal transplant recipients. In **chapter two**, we investigated habitual sodium intake in renal transplant recipients as compared to a healthy reference group and studied its association with blood pressure. In the general population, and in particular in CKD patients, dietary sodium is a well established risk factor for hypertension[21-25], a condition that is highly prevalent in renal transplant recipients as well [26]. We found habitual sodium intake to be considerably lower in renal transplant recipients than in healthy controls. Nevertheless, the majority

of patients still exceeded the daily amount as recommended in current international guidelines[27, 28]. Higher sodium intake was associated with higher systolic and diastolic blood pressure. Based on our data, it can be inferred that compliance with the maximally recommended daily intake would reduce mean systolic and diastolic blood pressure with approximately 5 mmHg and 3 mmHg respectively in our transplant population. These findings suggest that better control of sodium intake can lead to clinically relevant improvement of blood pressure in renal transplant recipients. However, a drawback of cross-sectional dietary studies is the firm and sometimes inextricable association between several nutrients. Indeed, in line with previous studies[29, 30], our study showed a close relation between sodium and protein. Both factors have been extensively studied for their potential influence on blood pressure in the general population and their joint presence in foods makes it difficult to distinguish between the effects of the separate dietary components on blood pressure. Therefore, in **chapter three** we had a closer look on the potential effects of dietary protein on blood pressure and renal function in renal transplant recipients. In non-transplant renal populations, dietary protein can affect renal haemodynamics as well as renal protein loss [31-33].

Concern exists that high protein intake induces high intraglomerular pressure and concurrent hyperfiltration, eventually leading to kidney damage and subsequent hypertension. In line, there is evidence that patients with chronic kidney disease benefit from a protein restricted diet [34, 35]. In our study among 625 renal transplant recipients, however, we did not find a continuous association of dietary protein with renal function or blood pressure, regardless of the type of protein (animal or protein). This was despite an average total protein intake of 1.1 g/kg/d, which exceeded the recommended values for renal patients (0.6-0.8 g/kg/d) [36]. Our study, therefore, did not support the hypothesis that high protein adversely influences renal function or blood pressure in RTR, at least within the range of the total protein intake we documented in our study population. One may hypothesize that potentially existing associations between protein intake and BP would go unnoticed as the majority of RTR use antihypertensive drugs. However, classical factors associated with BP in the general population such as age, gender and sodium intake (**chapter two**) were associated with BP in our RTR, which supports the power of our study to identify determinants of blood pressure in the current clinical context.

Despite the absence of an association between dietary protein and cardio-renal parameters, protein intake still might affect post-transplant conditions by contributing to metabolic parameters. Previous studies have shown that diet influences acid-base balance in the general population [37-40]. Dietary acid load originates from precursors like cationic amino acids and organic acids, while potassium salts of metabolizable anions, like citrate, have an alkalinizing effect [39, 41]. Since the kidney plays an important role in

acid-base homeostasis by excreting the excess of acids ingested [42-44], we hypothesized that renal transplant recipients, who obviously have a decreased capacity to excrete acid due to impaired renal function, might be at particular risk to develop diet induced acidosis and that they could benefit from dietary modifications to improve acid-base homeostasis.

In **chapter four**, we investigated whether metabolic acid load was related to acidosis in renal transplant recipients and whether dietary factors could be identified that influence metabolic acid load and subsequent acidosis. We found that of the 707 renal transplant recipients participating in our study, 31% had acidosis, defined as serum [HCO_3^-] lower than 24 mmol/L. In addition to conventional factors contributing to acidosis in renal transplant recipients, such as age and graft dysfunction, acidosis was also associated with urinary net acid excretion (NAE). Measurement of NAE is the gold standard for assessing metabolic acid load [45], and is based on 24 hour urinary excretion of titratable acid, ammonium and bicarbonate. The significant associations between NAE and serum [HCO_3^-] that we found, were consolidated by significant associations between acidosis and two validated algorithms estimating dietary acid load based on dietary recall (Potential Renal Acid Load (PRAL)[39] and Net Endogenous Acid Production (NEAP)[37]). PRAL and NEAP, and accordingly NAE, were higher in patients with higher intake of (animal) protein and calcium, and lower in patients with higher intakes of fruits and vegetables.

Based on the obtained data, it could be inferred that by rather simple dietary modification, about 5% of included renal transplant recipients with acidosis could achieve appropriate serum [HCO_3^-] levels. Based on these findings and given the high prevalence of acidosis long-term after renal transplantation, occasional venous blood gas analyses could aid in clinical practice. If acidosis is confirmed, medical practitioners should pay attention not only to known risk factors like graft function, but also to dietary habits since dietary intervention might improve long-term post-transplant outcomes.

The observed robust association between animal protein intake and acidosis in renal transplant recipients could very well point towards specific constituents of animal protein, rather than protein as a whole, contributing to acidosis. The sulfur containing amino acids (SAA), methionine and cysteine, which are known for their acidifying properties through conversion to sulfuric acid [38, 46], could account for our findings as they are predominantly found in animal protein. Indeed, we found significantly higher urinary sulfur excretion along with urinary net acid excretion. Considering the above, it might be suggested that renal transplant recipients should restrict intake of sulfur containing amino acids to improve acid-base homeostasis. On the other hand, SAA are the main substrate for endogenous synthesis of hydrogen sulfide (H_2S), which is a gaseous signaling molecule with substantial biological potential [47-50]. H_2S is suggested

to be beneficially involved in various (patho-)physiological processes including blood pressure regulation, inflammation, and cytoprotection during hypoxia which suggests a favorable role for SAA. These conflicting hypotheses on the role of sulfur were the focus of **chapter five**, where we explored the associations of the two most important urinary sulfur metabolites, sulfate and thiosulfate (TS), with cardiovascular and metabolic parameters in renal transplant recipients. Also, we analyzed the predictive capacity of both sulfur parameters for mortality. Sulfate was assumed to predominantly reflect dietary intake [51, 52], whereas TS is considered a specific intermediate of the H_2S metabolism [53]. Indeed, we observed a significant association of SAA intake with urinary sulfate, but not with thiosulfate. Remarkably, we found both urinary sulfate and urinary thiosulfate to be associated with a favorable cardio-metabolic risk profile in our renal transplant cohort. We also observed a significant predictive capacity of both sulfate and thiosulfate for incidence of mortality in our transplant cohort, also after adjustment for age, gender and graft function. Regarding TS, these findings were according to our primary hypothesis, since TS is assumed, at least in part, to reflect rate of endogenous H_2S synthesis. However, it might also be TS itself underlying the favorable association with cardiovascular parameters and patient survival since TS has been reported to exert protective activities in several pathophysiological processes as well, such as oxidative stress and vascular calcifications[54, 55]. In contrast, the beneficial associations of urinary sulfate with cardiovascular parameters and patient survival were quite puzzling, particularly because we observed significant association of urinary sulfate with metabolic acid load, which is suggested to have adverse effects on post-transplant conditions, as described in **chapter four**. Although it is difficult to conclude on causality in an observational study, a potential explanation for these associations might be that part of the excreted sulfate, like thiosulfate, has been incorporated in the H_2S synthesis. This would be in line with previous studies showing that systemically applied H2S is excreted as either TS or sulfate [56, 57]. Another intriguing finding in this study was that renal transplant recipients had a significantly higher excretion of thiosulfate compared with healthy subjects. This was quite surprising, since it might be expected that RTR, known to be at high cardiovascular risk compared to the general population, excrete less of an indicator of the allegedly favorable H_2S metabolism. Perhaps this higher TS excretion reflects a compensatory response, or up-regulation of H_2S and/or TS synthesis, to meet the increased demand for cardiovascular protection in renal transplant recipients. Obviously, long-term intervention studies with exogenous sulfur, either from the diet or pharmacological agents such as sodium thiosulfate, are warranted to clarify the exact role and underlying mechanisms of the sulfur metabolism in cardiovascular health in RTR. Notwithstanding the cross-sectional character of the study design, which warrants prudence in the drawing of firm conclusions, the results presented in **chapter**

five gave us the idea that 1) endogenously produced gaseous transmitters might play a significant role in long-term cardiovascular health after renal transplantation and 2) exogenous sources, e.g. from diet, might contribute to synthesis of these compounds, making dietary modification a valuable target in the management of post-transplant cardiovascular disease.

In **chapter six** we elaborated on these hypotheses and focused on nitric oxide (NO), another gaseous transmitter known to be involved in various physiological processes including regulation of vascular tone, adhesion of inflammatory cells and smooth muscle cell proliferation [58-60]. Some recent studies suggested that NO can be converted from exogenous inorganic nitrate, mainly originating from vegetables, and that nitrate supplementation may have therapeutic effects in renal and cardiovascular disease [61, 62]. Since NO itself can poorly be measured, as it has a short biological half-life, we estimated endogenous NO synthesis by measuring plasma and urinary nitrate and nitrate (NOx), which are stable end-products of NO and widely used as a marker of NO [63, 64]. Compared with healthy controls, renal transplant recipients had significantly lower urinary NOx-excretion despite similar intakes of vegetables. These lower levels could be explained by either impaired NO synthesis due to endothelial or graft dysfunction [65, 66], or excessive degradation as consequence of the continuous state of oxidative stress. Intriguingly, these urinary NOx-excretion levels were robustly associated with the extent of various cardiovascular risk factors, such as blood pressure, heart rate, hs-CRP and Nt-pro-BNP, even after adjustment for several confounders such as age, gender, renal function, medication use and vegetable intake, which is consistent with our hypothesis that hampered NO is directly connected to endothelial dysfunction. These results prompt for the evaluation of long-term administration of NO donors for treatment or even prevention of post-transplantation cardiovascular disease in renal transplant recipients. Despite similar intakes of vegetables in both healthy controls and renal transplant recipients, we found vegetable intake to be an independent determinant of urinary NOx excretion within our renal transplant cohort. The low urinary levels of NOx in our transplant cohort might therefore at least partly be explained by insufficient vegetable intake, which was far below the daily amounts as advocated by the universally adopted dietary approaches to stop hypertension (DASH) diet [67].

This restricted intake could be a relic of the rigid dietary regimen that has been prescribed to the majority of our transplant cohort during the dialysis episode preceding renal transplantation to prevent hyperkalemia. Importantly, a concomitant adverse effect of this regimen is the occurrence of deficiencies of nutrients predominantly present in vegetables, such as vitamin K. Vitamin K deficiency is increasingly recognized as a risk factor for cardiovascular morbidity and mortality in renal patients [68, 69]. An important function of vitamin K is the carboxylation, or biological activation, of matrix glutamate

protein (MGP), which inhibits vascular calcification [70, 71]. Poor vitamin K status, as a consequence of poor nutritional intake, results in high uncarboxylated MGP levels which is shown to be associated with vascular calcification. The combined observation of low vegetable intake and signs of endothelial dysfunction in our transplant population, might point toward vitamin K deficiency as underlying mechanism.

Therefore, in **chapter seven** we focused on the association of vitamin K intake with vascular vitamin K status in renal transplant recipients. In contrast with all other studies described in this thesis, we obtained data on vitamin K intake from dietary diaries instead of food frequency questionnaires. Data from diaries in general is more accurate and gives a better reflection of the intake over de preceding days. However, keeping a diary by patients on one hand, and processing the data by the researcher on the other, is very labor-intensive and time consuming [72]. Therefore, for logistic reasons, we obtained data on vitamin K intake from a small subgroup (n=60) of our renal transplant cohort. We found that dietary vitamin K intake was below recommendations in 50% of our renal transplant cohort. Even more important was that vascular vitamin K status was insufficient in the majority of renal transplant recipients, reflected by elevated plasma uncarboxylated MGP levels. In addition, we observed that renal transplant recipients with vitamin K insufficiency were more likely to experience low-grade inflammation, which itself is supposed to contribute to or reflect increased cardiovascular risk after renal transplantation [73-75]. Whether increased intake of vitamin K influences cardiovascular calcifications, either via improvement of the inflammatory state should best be proven in randomized controlled prospective intervention studies.

Discussion

In this thesis, we studied the dietary patterns of renal transplant recipients long-term after transplant surgery. Second we investigated the association of several nutrients with various cardiovascular, metabolic and renal risk parameters in our search for modifiable dietary components that might contribute to improved outcomes after renal transplantation.

In comparison with the available general guidelines for a good clinical health, renal transplant recipients deviated significantly. With respect to nutrients, intake of sodium, protein, phosphorus and saturated fat were higher than advocated, whereas intake of poly-unsaturated fats, carbohydrates and fiber were lower than the daily recommended amounts. In comparison with a healthy reference group consisting of potential living kidney donors, absolute intake of nutrients was lower in renal transplant recipients, which for the first time became visible in chapter two where 24-hour urinary sodium excretion was 20% lower compared with healthy controls. We concluded that renal transplant recipients apparently were more compliant to sodium restricted diets, likely as a result of intense dietary counseling during the course of renal disease and post-transplantation follow-up. However, when additional dietary data became available in subsequent studies, described in chapter four, five and six, we found out that it was primarily the absolute, or caloric intake that was lower rather than the composition of the diet. One could hypothesize that RTR would give desirable answers when filling in a dietary questionnaire, rendering biased results. However, comparison of data obtained from FFQ with those obtained from urinary urea excretion, the latter obviously resistant to pleasing behavior, yielded similar results.

Despite the lower and seemingly adequate total dietary intake in RTR, we observed a contradictory average body mass index over 26.5 kg/m^2 which, according to the WHO guidelines, is defined as overweight. Potential explanations for these paradoxical findings might be the sedentary lifestyle which is commonly seen after renal transplantation. Other explanations might be an altered metabolism due to long-term use of immunosuppressive drugs, which have been shown to contribute to diabetes and dyslipidemia. Additional adverse effects of these agents include changes of intestinal flora and, as a potential consequence, an altered absorption and handling of nutrients. A third factor potentially underlying these conflicting phenomena is an increased extracellular volume as a result of fluid retention caused by corticosteroids and renal impairment or cardiac failure that is not compensated for by diuretics. A fourth potentially underlying factor could be a difference in muscle mass, which likely was present, as reflected by the difference in 24h urinary creatinine excretion between RTR and healthy controls. Irrespective of the mechanism underlying the discrepancy

of intake and BMI, our findings support our primary hypothesis that it is not merely caloric intake that plays a role in post-transplant conditions, but also diet composition. Indeed, in all our statistical analyses on the associations of dietary factors with health parameters in our transplant cohort, our findings remained essentially unchanged after adjustment for body composition, which even more points towards the quality of the diet that should receive proper attention.

With one single exception, the studies described in this thesis have a cross-sectional design and therefore are subject to certain limitations. One of the drawbacks of cross-sectional data is that both intake of dietary factors and the outcome variables are assessed at the same moment in time, which makes it difficult to address the temporality of the associations we found. It is possible that renal transplant recipients that perceived increased cardiovascular, metabolic or renal risk changed their overall dietary habits, either themselves or after medical advice, resulting in erroneous conclusions. However, we consider intentional dietary changes in renal transplant recipients unlikely, since many of the outcome parameters we investigated, such as acid-base-parameters, renal function and low-grade inflammation are often asymptomatic, and do not lead to changes in dietary recommendations. Additionally, in clinical practice, most of these symptoms are considered present as a matter of course, and also are more likely to be treated with pharmacological agents than with dietary intervention. Another limitation that applies to the majority of our studies is the use of questionnaires that depend on adequate dietary recall of our patients. Although the use of questionnaires is a generally accepted tool for the inquiry of foods and beverages in epidemiological studies, some misclassification might still occur. To verify the data obtained from food frequency questionnaires, we validated those against 60 dietary diaries or, if possible, against urinary excretions. We found correlations that were comparable with those observed in previous studies analyzing validity of FFQs in population-based cohort studies. Nevertheless, some random misclassification of dietary intake remains inevitable, though it would have weakened the observed associations between dietary factors and clinical parameters rather than that they spuriously would have become into existence.

Future Perspectives and Clinical Implications

We showed in several cross-sectional studies that dietary intake is associated with various post-transplant conditions such as blood pressure, acid-base homeostasis and low-grade inflammation, all very prevalent in renal transplant recipients. A quick search on Pubmed learns that hardly any data are available yet on the role of nutrition long-term after transplant surgery. Considering the cross-sectional design of our studies and its inherent limitations, our findings constitute dangerous ground to jump to firm conclusions. It goes without saying that our findings and conclusions require further consolidation and confirmation. It would be informative to investigate in a prospective study whether the nutritional factors under study in this thesis also predict hard end-points such as cardio-vascular events, graft failure or patient mortality. In chapter five we had a first look on the association of sulfur metabolites, partly reflecting intake of sulfur containing amino acids, with incidence of mortality in renal transplant recipients and found sulfate and thiosulfate to be significant predictors for overall death long-term after transplantation. These promising longitudinal data support our cross-sectional findings of the association between sulfur metabolites and a favorable cardiovascular risk profile in RTR. Nevertheless, despite a consolidation of the beneficial relation between sulfur metabolites and post-transplant conditions, the observational nature of these longitudinal data still calls prudence to drawing firm conclusions as it provides no conclusive evidence for the protective potential of exogenous sulfur.

An elegant way to investigate the effects of sulfur on cardiovascular health and patient survival would be to perform a prospective intervention study in renal transplant recipients. In the study described in chapter five, an association of urinary sulfate and thiosulfate was observed with incidence of mortality within a relatively short period of follow-up (median 17 months), indicating that in an intervention study, with even larger contrasts in exposure, the potential effects of exogenous sulfur on hard outcome variables might be detected within a foreseeable amount of time. If such a study would be performed, taking for granted that time and financial measures are at unlimited disposal, a large, multi-center randomized controlled trial in stable renal transplant recipients would be the study of choice. The trial could include three groups of patients with one control group receiving a standard Western diet. A second group could receive a diet rich in methionine and cystein containing foods, such as poultry, oats and eggs. In prior analyses, intake of sulfur containing amino acids correlated well with urinary sulfate excretion, indicating that habitual diet indeed contributes to the systemic sulfur pool. The third study group could receive a Western diet, like the control group, however, with additional daily oral application of sodium thiosulfate as principal source of sulfur. Sodium thiosulfate is not yet registered; however, it is widely used as antidote to cyanide

poisoning or as treatment of calciphylaxis in hemodialysis patients with end stage renal disease and is well tolerated. Adherence to the interventions could be assessed by dieticians and by biochemical measurements during monthly visits to the outpatient clinic. Outcome variables in this study could include change, from baseline to the end of intervention, in the cardiovascular and metabolic parameters as investigated in chapter five, with specific focus on hemodynamics, vascular calcification status and inflammation.

If the findings in this thesis are confirmed by longitudinal data or preferably, by long-term intervention studies, this could have several clinical implications. Awareness should be raised among renal transplant recipients as well as among clinicians on diet composition and on its role in post-transplant physical health. During follow-up, attention should be directed not only to conventional factors contributing to post-transplant cardiovascular and metabolic morbidity, such as graft function, but also to nutritional habits of our patients. Regular involvement of a dietician at the outpatient clinic could prove to be useful in helping our patients to gain insight in their dietary habits and to achieve an optimal diet. Even slight dietary modifications might already improve long-term morbidity in renal transplant recipients and improve long-term graft and even patient survival.

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Nederlandse samenvatting

Wereldwijd is het aantal patiënten met eindstadium nierfalen in de afgelopen decennia gestaag toegenomen. Nederland telt momenteel ruim 16.000 patiënten met nierfalen, wat neerkomt op 1 patiënt per 1000 inwoners. De belangrijkste oorzaak voor deze stijging ligt in de toename van leefstijl gerelateerde verschijnselen als hypertensie, overgewicht en diabetes mellitus type 2, die alle erkende risicofactoren vormen voor de ontwikkeling van nierschade.

Het merendeel van de patiënten met nierfalen start met nierdialyse als nierfunctievervangende therapie. Hoewel hierdoor de levensverwachting duidelijk wordt verbeterd, is het sterftecijfer onder dialysepatiënten nog altijd erg hoog; na aanvang van nierdialyse is slechts de helft van alle patiënten na drie jaar nog in leven.

Voor patiënten met eindstadium nierfalen vormt niertransplantatie de behandeling van eerste keuze. Niet alleen maakt het nierdialyse overbodig en verbetert het daarmee de kwaliteit van leven, ook is de levensverwachting van niertransplantatiepatiënten vele malen beter ten opzichte van die van dialysepatiënten. Mede dankzij de introductie van nieuwe afweeronderdrukkende medicijnen komt acute afstoting van de transplantaatnier steeds minder vaak voor. Toch is op lange termijn ook niertransplantatie niet zaligmakend. Zelfs na een succesvolle niertransplantatie gaat de helft van alle getransplanteerde nieren 12 tot 25 jaar na transplantatie verloren. Een van de oorzaken hiervoor is het feit dat een groot aantal niertransplantatiepatiënten komt te overlijden met een nog functionerende transplantaatnier. Hart- en vaatziekten, waaronder hypertensie, vaatverkalking en schade aan de binnenbekleding van bloedvaten, vormen de belangrijkste doodsoorzaak bij niertransplantatiepatiënten en de mortaliteit ten gevolge hiervan is vijfmaal hoger in vergelijking met de algemene bevolking. Het is om die reden van cruciaal belang dat er gezocht wordt naar mogelijkheden om hart- en vaatziekten na niertransplantatie te voorkomen en daarmee de langetermijnoverleving van niertransplantatiepatiënten te verbeteren.

In de afgelopen decennia is veel onderzoek gedaan naar de relatie tussen verschillende voedingselementen en het cardiovasculair risico in de algemene bevolking. Veranderingen van leefstijl, waaronder de aanpassing van voedingsgewoonten, zijn effectief gebleken in het voorkómen van hart- en vaatziekten en dragen daarmee bij aan een gezondere overleving. Wat de rol van voeding is bij niertransplantatiepatiënten is tot op heden nooit goed onderzocht. Omdat niertransplantatiepatiënten om uiteenlopende redenen een zeer specifieke populatie vormen, kunnen resultaten uit onderzoeken in de algemene bevolking niet een op een worden geëxtrapoleerd naar deze patiëntengroep. Wegens het gebrek aan wetenschappelijk bewijs betreffende de rol van voeding in de gezondheid van niertransplantatiepatiënten, zijn er voor hen tot op heden geen gefundeerde voedingsrichtlijnen beschikbaar. Voor zowel medici als voor transplantatiepatiënten is het daarom onduidelijk wat de optimale samenstelling

van het dieet zou moeten zijn ter verbetering van de cardiovasculaire gezondheid van niertransplantatiepatiënten.
Een van de doelen van dit proefschrift is om de voedingsgewoonten van een groot cohort niertransplantatiepatiënten in kaart te brengen en deze te vergelijken met die van gezonde personen. Daarnaast onderzoeken we wat de relatie is van verschillende voedingsfactoren met het verhoogde risico op hart- en vaatziekten bij niertransplantatiepatiënten, opdat handvatten worden verkregen om uiteindelijk gerichte interventies te kunnen verrichten om de hoge morbiditeit en mortaliteit onder niertransplantatiepatiënten te verlagen. Voor dit onderzoek hebben 707 niertransplantatiepatiënten, die allen in het Universitair Medisch Centrum Groningen hun niertransplantatie hebben ondergaan, de bereidheid getoond om mee te werken.

Om een beeld te krijgen van de voedingsgewoonten van deze niertransplantatiepatiënten hebben we gebruik gemaakt van twee verschillende methoden. Ten eerste hebben we aan alle patiënten voedingsvragenlijsten, zogenaamde 'Food Frequency Questionnaires' voorgelegd. Dit zijn wereldwijd erkende vragenlijsten die een beeld geven van de voeding over de laatste maanden. Met name voor het analyseren van de inname van macronutriënten, zoals eiwit, is deze methode geschikt, vooral in grote cohorten. Ten tweede hebben we alle niertransplantatiepatiënten gedurende 24 uur al hun urine laten verzamelen. Deze 24-uurs urine, mits zorgvuldig verzameld, vormt een 'vingerafdruk' van de voeding die iemand recent heeft binnengekregen. Deze methode, hoewel arbeidsintensiever dan het invullen van een vragenlijst, heeft de voorkeur wanneer het gaat om het analyseren van bijvoorbeeld zoutinname, omdat het zout dat in de urine gemeten wordt, onder normale omstandigheden nagenoeg gelijk is aan de hoeveelheid die met de voeding het lichaam is binnengekomen. Waar mogelijk hebben we de resultaten van beide meetmethoden onderling vergeleken, ter analyse van de consistentie en validiteit van onze onderzoeksresultaten.

In hoofdstuk twee hebben we onderzocht hoeveel zout niertransplantatiepatiënten gebruiken en in hoeverre zoutinname bij hen geassocieerd is met bloeddruk. In de algemene bevolking is een hoge zoutinname een bekende risicofactor voor hypertensie, maar of het ook bij niertransplantatiepatiënten bijdraagt aan een hoge bloeddruk is nooit onderzocht. We zagen dat niertransplantatiepatiënten significant minder zout gebruiken dan personen uit een gezonde controlegroep, maar desondanks veel meer dan in de richtlijnen voor de algemene bevolking wordt geadviseerd. Een hogere zoutinname was significant geassocieerd met een hogere bloeddruk. Uit onze studieresultaten kan worden afgeleid dat niertransplantatiepatiënten, indien ze zich zouden houden aan de aanbevolen hoeveelheid zout, een beduidend lagere bloeddruk zouden

hebben. Daarmee zou het risico op hart- en vaatziekten kunnen worden beperkt. Een schaduwzijde van bovengenoemde studie is het feit dat het eten van meer zout veelal gepaard gaat met de intake van andere voedingsstoffen. Zo hebben eerdere studies aangetoond dat mensen die veel zout eten, automatisch ook veel eiwitten binnenkrijgen door het gezamenlijk voorkomen in voeding.

Om die reden hebben we in hoofdstuk drie de inname van eiwitten apart onder de loep genomen. Het is bekend dat voedingseiwitten invloed uitoefenen op de bloeddoorstroming van de nier en daarmee kunnen bijdragen aan nierschade, juist bij nierpatiënten. In lijn daarmee is het feit dat patiënten met chronische nierziekten baat hebben bij een eiwitarm dieet. In niertransplantatiepatiënten vonden we hiervoor echter geen aanwijzingen. Nierfunctie noch bloeddruk waren geassocieerd met de inname van eiwitten, ongeacht de bron van eiwit, terwijl de gemiddelde inname van eiwit ver boven de geadviseerde hoeveelheid bij nierpatiënten lag. Onze studie suggereert daarmee dat voedingseiwitten, zij het binnen de range die we gedocumenteerd hebben in onze studiepopulatie, geen nadelige gevolgen hebben op nierfunctie en bloeddruk. Hoewel geopperd zou kunnen worden dat eventuele gevolgen van eiwitten op de bloeddruk teniet worden gedaan door de grote hoeveelheden bloeddrukverlagende middelen die door niertransplantatiepatiënten worden gebruikt, zagen we dat bekende risicofactoren, zoals leeftijd, geslacht en zoutinname, wel degelijk geassocieerd waren met bloeddruk in onze onderzoekspopulatie.

Het is overigens niet gezegd eiwitten helemaal niet van invloed zijn op de gezondheid van niertransplantatiepatiënten. Voedingseiwitten hebben namelijk ook invloed op het zuur-base evenwicht doordat ze opgebouwd zijn uit onder andere aminozuren die bijdragen aan de metabole zuurbelasting van het lichaam. Aangezien de nieren een belangrijke rol spelen bij het uitscheiden van een teveel aan zuur, veronderstelden we dat niertransplantatiepatiënten, ten gevolge van hun verminderde nierfunctie, een verhoogd risico lopen op metabole acidose, ten dele geïnduceerd door het dieet. In hoofdstuk vier onderzochten we of metabole zuurbelasting door de voeding daadwerkelijk gerelateerd was aan metabole acidose en of er voedingsfactoren konden worden geïdentificeerd die acidose beïnvloeden. Van de 707 niertransplantatiepatiënten had 31% een metabole acidose, blijkens een bicarbonaat concentratie in het bloed die lager was dan 24 mmol/L. Aan de hand van de 24-uurs urine hebben we bij alle patiënten de metabole zuurbelasting (Net Acid Excretion; NAE) gemeten volgens de gouden standaard. We zagen dat de mate van acidose significant geassocieerd was met de NAE en dat patiënten met een grotere inname van (dierlijke) eiwitten en calcium een significant grotere kans hadden op acidose dan patiënten die veel groenten en fruit aten. De geobserveerde samenhang tussen eiwitten en metabole acidose in niertransplantatiepatiënten zou verklaard kunnen worden door specifieke

bestanddelen van eiwitten, in plaats van eiwitten in hun geheel. Zo zouden bijvoorbeeld de zwavelbevattende aminozuren, methionine and cysteine, die veel voorkomen in dierlijke eiwitten, verantwoordelijk kunnen zijn voor de verzurende werking van eiwitten door omzetting naar sulfaat. Dit suggereert dat niertransplantatiepatiënten geadviseerd moet worden de inname van zwavelbevattende eiwitten te beperken. Aan de andere kant vormt zwavel een belangrijk bestanddeel van sulfidegas (H_2S), dat juist een gunstige werking heeft op bloeddrukregulatie, ontstekingsremming en weefselbehoud tijdens lage zuurtstofdruk. Deze tegenstrijdigheid vormde de basis voor de studie zoals beschreven in hoofdstuk vijf waarin we de samenhang bestudeerden tussen de belangrijkste zwavelhoudende metabolieten (sulfaat en thiosulfaat) en cardiovasculaire en metabole parameters. Ook hebben we gekeken naar de voorspellende waarde van deze zwavelhoudende stoffen voor sterfte. Sulfaat is hierbij een reflectie van de inname van zwavelbevattende eiwitten, terwijl thiosulfaat een intermediair is in het H_2S-metabolisme. Dit laatste bleek ook uit de significante associatie tussen inname van zwavelbevattende eiwitten en het urine sulfaatgehalte, die niet gevonden werd tussen zwavelbevattende eiwitten en het urine thiosulfaatgehalte. Opvallend was dat zowel urinesulfaat als urinethiosulfaat geassocieerd was met een gunstig cardiovasculair risicoprofiel. We vonden zelfs dat de concentraties van zowel sulfaat als thiosulfaat in de urine van voorspellende waarde waren voor sterfte, waarbij een hogere waarde in de urine tot minder sterfte leidt. Voor thiosulfaat was dit volgens onze primaire hypothese, aangezien thiosulfaat, in meer of mindere mate, een reflectie is van de endogene synthese van H_2S. De gevonden gunstige associatie tussen urine sulfaat en sterfte was echter haaks op onze primaire hypothese, vooral omdat we een significante associatie vonden tussen de inname van zwavelbevattende eiwitten en NAE, zoals beschreven in hoofdstuk vier. Een mogelijke verklaring hiervoor zou kunnen zijn dat een deel van het uitgescheiden sulfaat, net als het thiosulfaat, ingelijfd is geweest in de H_2S-synthese. Longitudinale interventiestudies met exogeen zwavel, uit de voeding dan wel uit farmacologische preparaten, zullen uitsluitsel moeten geven over de daadwerkelijke rol, en het onderliggende mechanisme, van het zwavelmetabolisme in de cardiovasculaire gezondheid van niertransplantatiepatiënten. Niettegenstaande de cross-sectionele studieopzet van hoofdstuk vijf, deden de verkregen resultaten ons veronderstellen dat niet alleen endogeen geproduceerde ‘gasotransmitters’ zoals H_2S een belangrijke rol zouden kunnen spelen in cardiovasculaire gezondheid na niertransplantatie, maar ook dat voeding van belang zou kunnen zijn bij de synthese van deze gasverbindingen. In hoofdstuk zes zijn we hierop verder gegaan waarbij we hebben gekeken naar stikstofgas (NO) dat ook zijn gunstige werking heeft bewezen in verschillende fysiologische processen zoals de regulatie van de vaatweerstand, ontstekingsprocessen en de celdeling van gladde spiercellen. Eerdere studies hebben

gesuggereerd dat NO kan worden omgezet uit anorganisch nitraat, dat veel voorkomt in groenten, en dat nitraatsuppletie therapeutische effecten heeft bij patiënten met nierziekten of hart- en vaatziekten. Omdat NO-gas vluchtig is en derhalve niet goed kan worden gemeten, hebben we de restproducten van NO, nitriet- en nitraat (NO_x), in de urine als maat genomen voor het endogeen gevormde NO, zoals dat wereldwijd ook wordt gedaan. Het urine NO_x-gehalte was sterk en gunstig geassocieerd met verschillende cardiovasculaire risicofactoren zoals bloeddruk, hartslag, ontsteking en hartfalen. Deze bevindingen stroken met de hypothese dat verminderde NO-synthese direct verband houdt met verminderde endotheelfunctie. Verder zagen we dat de inname van groenten een onafhankelijke determinant was van het urine- NO_x-gehalte, maar ook dat de hoeveelheid groenten die niertransplantatiepatiënten dagelijks eten ver beneden de aanbevolen hoeveelheid lag. De resultaten van deze studie suggereren dat met het verhogen van de inname van groenten een gunstiger cardiovasculair risicoprofiel zou kunnen worden verkregen, mogelijk dankzij een verhoging van de endogene NO-synthese.

De in hoofdstuk zes vastgestelde lage inname van groenten door niertransplantatie-patiënten zou een overblijfsel kunnen zijn van het strenge dieetregime dat voor de meerderheid van de patiënten gold tijdens nierdialyse voorafgaand aan de niertransplantatie, ter voorkoming van hyperkaliëmie. Inherent aan dit strenge 'dialysedieet' is echter het risico op deficiënties van verschillende nutriënten die ook in groenten aanwezig zijn, zoals vitamine K. Een vitamine K tekort wordt steeds meer erkend als een risico voor het verhoogde risico op hart- en vaatziekten in nierpatiënten. Een belangrijke rol voor vitamine K is weggelegd in de remming van vaatverkalking door carboxylering, ofwel activatie, van het beschermende 'matrix glutamate protein' (MGP). Bij een lage inname van vitamine K, wordt er minder MGP gecarboxyleerd waardoor sneller vaatverkalking zal optreden. Het gecombineerd voorkomen van een lage inname van groenten en de hoge prevalentie van verminderde endotheelfunctie bij niertransplantatiepatiënten zou derhalve gelegen kunnen zijn in een vitamine K deficiëntie als onderliggend mechanisme. In hoofdstuk zeven zagen we dat in de helft van de niertransplantatiepatiënten de vitamine K inname lager was dan de aanbevolen dagelijkse hoeveelheid. Daarbij zagen we dat er een verhoogde plasmaconcentratie ongecarboxyleerd MGP werd gevonden in het grootste deel van de niertransplantatiepatiënten. Patiënten die een tekort aan vitamine K hadden, hadden een grotere kans op laaggradige ontsteking, een fenomeen dat op zich ook weer een risico vormt voor hart- en vaatziekten na transplantatie. Of een toename van de inname van vitamine K daadwerkelijk zal bijdragen aan verminderde vaatverkalking, zal echter onderzocht moeten worden met gerandomiseerde, gecontroleerde, prospectieve interventiestudies.

De bevindingen uit de studies zoals beschreven in dit proefschrift suggereren dat de samenstelling van het dagelijks dieet een rol speelt in de gezondheid van niertransplantatiepatiënten. Desalniettemin is voorzichtigheid geboden bij het trekken van stevige conclusies. Het moge duidelijk zijn dat meer maar vooral prospectieve interventiestudies nodig zijn om te onderzoeken of de voedingsfactoren zoals deze aan bod zijn gekomen in dit proefschrift ook van voorspellende waarde zijn voor harde eindpunten zoals cardiovasculaire complicaties, transplantaatoverleving en sterfte. Mocht inderdaad blijken dat er winst in patiënt- dan wel transplantaatoverleving is te behalen door modificatie van voedingsgewoonten van niertransplantatiepatiënten, dan zal dit duidelijk consequenties hebben voor de invulling van de follow-up van deze patiëntengroep. Naast aandacht voor de medicamenteuze benadering van het cardiovasculaire risico van niertransplantatiepatiënten, zal ook nadruk moeten komen te liggen op de samenstelling van het dieet van niertransplantatiepatiënten ter bevordering van langetermijnoverleving. Regelmatige betrokkenheid van een voedingsdeskundige op de polikliniek zou hierbij van grote waarde kunnen zijn.

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